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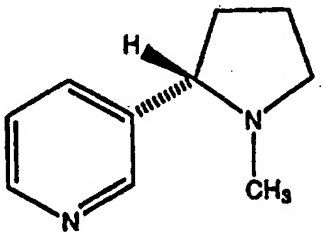
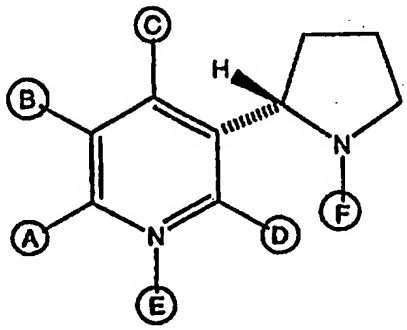
(54) Title: HAPTEN–CARRIER CONJUGATES FOR USE IN DRUG–ABUSE THERAPY AND METHODS FOR PREPARATION OF SAME

(57) Abstract

Hapten–carrier conjugates capable of eliciting anti–hapten antibodies *in vivo* are described. Methods of preparing said conjugates and therapeutic compositions are also described. Where the hapten is a drug of abuse, a therapeutic composition containing the hapten–carrier conjugate is particularly useful in the treatment of a drug addiction. The therapeutic composition is suitable for co–therapy with other conventional drugs. Passive immunization using antibodies raised against the hapten–carrier conjugates of the invention are also described.





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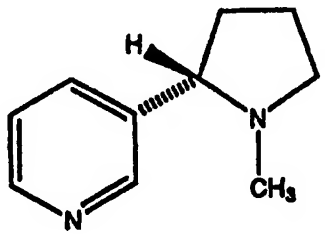

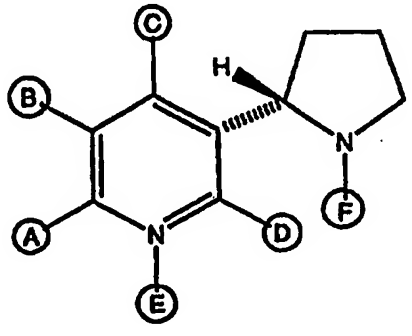

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HAPTEN-CARRIER CONJUGATES FOR USE IN DRUG-ABUSE THERAPY AND METHODS FOR PREPARATION OF SAME

FIELD OF THE INVENTION

The present invention relates to treatment of drug abuse. More specifically, the present invention relates to methods of treating drug abuse using drug/hapten-carrier conjugates which elicit antibody responses and/or using the antibodies to the drug/hapten-carrier conjugates.

BACKGROUND OF THE INVENTION

The prevalence of drug use and abuse worldwide, especially in the United States, has reached epidemic levels. There are a plethora of drugs, both legal and illegal, the abuse of which have become serious public policy issues affecting all strata of society with its obvious medical and social consequences. Some users live in an extremely high risk population associated with poverty and illegal activity. Other users who might classify themselves as recreational users are at risk due to (a) properties of the drug(s) which make them addictive, (b) a predisposition of the user to become a heavy user or (c) a combination of factors including personal circumstances, hardship, environment and accessibility. Adequate treatment of drug abuse, including polydrug abuse, requires innovative and creative programs of intervention.

Three especially problematic drugs of addiction are cocaine, heroin and nicotine. Cocaine is an alkaloid derived from the leaves of the coca plant *(Erythroxylon coca)*. In the United States alone, there currently are more than 5 million regular cocaine users of whom at least 600,000 are classified as severely addicted (Miller et al. (1989) *N.Y. State J. Med.* pp. 390-395; and Carroll et al. (1994) *Pharm. News.* 1:11-16). Within this population, a significant number of addicts actively are seeking therapy. For example, in 1990, 380,000 people sought medical treatment for cocaine addiction and the number is increasing. At that time, it was estimated that 100,000 emergency room admissions per year involve cocaine use. The cumulative effects of cocaine-associated violent crime, loss in individual productivity, illness, and death is an international problem.

The lack of effective therapies for the treatment of cocaine addiction strongly suggests that novel approaches must be developed. Additional factors contributing to the lack of successful treatment programs is that patterns of cocaine abuse have varied with time. In an article entitled "1994 Chemical Approaches to the Treatment of Cocaine Abuse" (Carroll et al. (1994) *Pharm. News*, Vol. 1, No. 2), Carroll et al. report that since the mid-1980's, intravenous and nasal dosing of the hydrochloride salt (coke, snow, blow) and smoking of cocaine free-base (crack) have become common routes of administration, producing euphoria and psychomotor stimulation which last 30-60 minutes. Unlike some other abused drugs, cocaine can be taken in binges lasting for several hours. This behavior leads to addiction, and in some cases, to toxic consequences (Carroll et al., *Pharm. News*, supra.)

There are only very limited treatments for drugs of abuse and no effective long term treatments for cocaine addiction. Treatments include, but are not limited to, counseling coupled with the administration of drugs that act as antagonists at the opioid receptors or drugs that try to reduce the craving associated with drug addiction. One approach to treatment is detoxification. Even temporary remissions with attendant physical, social and psychological improvements are preferable to the continuation or progressive acceleration of abuse and its related adverse medical and interpersonal consequences (Wilson et al. in *Harrison's Principle of Internal Medicine* Vol. 2, 12th Ed., McGraw-Hill (1991) pp. 2157-8). More specifically, pharmacological approaches to the treatment of cocaine abuse generally involve the use of anti-depressant drugs, such as desipramine or fluoxetine which may help manage the psychological aspects of withdrawal but, in general, do not directly affect the physiology of cocaine. (Kleber (1995) *Clinical Neuropharmacology* **18**:96-109). Further, their effectiveness varies widely (Brooke et al. (1992) *Drug Alcohol Depend.* **31**:37-43). In some studies, desipramine reduced self-administration (Tella (1994) *College on Problems of Drug Dependence Meeting Abstracts;* Mello et al. (1990) *J. Pharmacol. Exp. Ther.* **254**:926-939; and Kleven et al. (1990) *Behavl. Pharmacol.* **1**:365-373), but abstinence rate following

treatment did not exceed 70% (Kosten (1993) *Problems of Drug Dependence, NIDA Res. Monogr.* 85). There has also been the use of drugs which potentiate dopaminergic transmission, such as bromocriptine, but the benefits of such drugs are limited in part by toxicity (Taylor et al. (1990) *West. J. Med.* **152**:573-577). New drugs aimed at replacing methadone for opioid addiction, such as buprenorphine, have also been used based on cross-interference with the dopaminergic system, however only limited clinical study information is available (Fudula et al. (1991) *NIDA Research Monograph,* **105**:587-588). Buprenorphine has been reported to decrease cocaine self- administration (Carroll et al. (1991) *Psychopharmacology* **106**:439-446; Mello et al. (1989) *Science* **245**:859-862; and Mello et al. (1990) *J. Pharmacol. Exp. Ther.* **254**:926-939); however, cocaine abstinence rates following treatment generally do not exceed 50% (Gastfried et al. (1994) *College on Problems of Drug Dependence Meeting Abstracts;* and Schottenfeld et al. (1993) *Problems on Drug Dependence, NIDA, 10 Res. Monogr.* 311).

Present therapies used to treat cocaine addicts have at least four major limitations leading to a very high rate of recidivism. First, and perhaps most fundamentally, the contributing neurochemical events in cocaine abuse and addiction are complex (Carroll et al. (1994) *supra.*). As a result, single acting neuropharmacological approaches, such as inhibition of dopamine uptake, do not appear to be sufficient to overcome addiction. Second, the drugs currently used in cocaine addiction treatments have significant side-effects themselves, limiting their utility. Third, drug therapy compliance is problematic among this patient population. Current therapies can require frequent visits to a health care provider and/or self-administration of drugs designed to cure the addict of his habit. Because many of these drugs prevent the euphoria associated with cocaine, there is a strong disincentive to taking the drug. (Carroll, et al. (1994) *supra.*; Kosten et al. (1993) Problems of Drug Dependence, *NIDA Res. Monogr.* **132**:85; Schottenfeld et al. (1993) Problems of Drug Dependence, *NIDA Res. Monogr.* **132**:311.) Fourth, because of the complex chemistries involved in pharmacological therapies, many of them may be incompatible with other therapies currently in use or in clinical

trials. Finally, most of the pharmacotherapy studies have been administered in context of low-intensity outpatient treatment programs and have not been linked with intensive outpatient treatment or other psychosocial treatment that appears necessary for successful management of cocaine dependent patients. (Rao (1995) *Psychiatric Annals* **25(6)**:363-368).

Heroin is an opioid drug which is derived from the opium poppy. It is the most commonly abused opioid drug and is readily available on the illicit market in the United States. The quality of heroin which is currently available to addicts is high (45-80% purity), leading to a greater level of physical dependance than in previous years. The more potent forms of heroin are usually administered by smoking or snorting, making the initiation more accessible to people who are averse to intravenous injection, which is the usual form of administration. The mortality amongst heroin abusers is high. Early death comes from a variety of situations, eg, severe bacterial infections and HIV from shared injection paraphernalia. There is no accurate record of the number of heroin abusers in the United States, but an estimation based on extrapolating overdose deaths, addicts arrested and those applying for treatment, puts the numbers between 750,000 and 1 million.

Injection of heroin solution produces a rapid onset of variety of pleasurable sensations, including warmth, taste and a high (comparable to orgasm). Heroin is rapidly hydrolyzed to 6-monoacetyl morphine (6-MAM) and then to morphine. Both heroin and 6-MAM are highly lipid soluble and cross the blood-brain barrier more readily than morphine. There is an initial state of euphoria which lasts from between 45 seconds to several minutes, followed by a period of tranquility, which lasts from 3 to 5 hours depending upon the dose. After this, there follows withdrawal symptoms when the addict becomes irritable and aggressive, and has a general feeling of "sickness".

Current treatment usually involves pharmacological intervention to treat withdrawal signs and symptoms. Detoxification usually begins by dosing with a

long acting opioid such as methadone. Another approach is the use of the hypertensive drug clonidine, which alleviates many of the symptoms of withdrawal, but not the craving and generalized aches which are characteristic. As with most addictions, there is a high rate of recidivism. Long term management of patients and stabilization on methadone are the most successful treatments to date, requiring vigilance and compliance.

Nicotine (1-Methyl-2- (3-pyridyl)pyrrolidine) is an alkaloid derived from the tobacco leaf. Nicotine use is widespread throughout the world and is legally available in many forms such as cigarettes, cigars, pipe tobacco, and smokeless (chewing) tobacco. Although the addictive nature of nicotine and the dangers of smoking have been known for many years (Slade et al. (1995) *JAMA* 274(3):225-233), cigarette smoking remains popular. An estimated 51 million Americans smoke and, according to the Center for Disease Control and Prevention, 420,000 people each year die from smoking related disorders.

The most popular nicotine delivery system is the cigarette. Cigarettes contain 6 to 11 mg of nicotine, of which the smoker typically absorbs 1 to 3 mg. The typical pack-per-day smoker absorbs 20 to 40 mg of nicotine each day, achieving plasma concentrations of 25 to 50 ng per milliliter. The plasma half life of nicotine is approximately two hours; the half life of the major metabolite cotinine is 19 hours. (Henningfield (1995) *The New England Journal of Medicine* 333(18) :1196-1203).

Since nicotine is legally and widely available there is relatively low pressure against its use, unlike cocaine and heroin. Although a large percentage of addicted smokers have expressed a desire to stop smoking, and many actually try to stop, only 2 to 3 percent of smokers become nonsmokers each year. (Henningfield (1995) supra.). The high rate of recidivism in smokers who try to quit is indicative of the strong effect of nicotine dependence. (O'Brien et al. (1996) *Lancet* 347:237-240).

Nicotine addiction is a chronic, relapsing disorder. Nicotine targets the mesolimbic reward system eventually resulting in physiological dependence. Evidence suggests that nicotine binds to the α-subunit of the nicotinic acetylcholine receptors in the central and peripheral nervous systems resulting in increased dopamine release. It is thought that increased numbers of nicotinic acetylcholine receptors in the brain enhance the physiological dependence of nicotine (Balfour (1994) *Addiction* 89:1419-1423). These physiological effects of nicotine are powerful reinforcers of the psychological addiction. The nicotine users increased cognition and improved mood, as well as the negative effects associated with abstinence (i.e., withdrawal symptoms), serve as powerful motivators for continued tobacco use.

The lack of effective therapies for nicotine dependence and the poor rate of success in those who try and quit its use indicate that there is a strong need for a new therapy. Currently, the two most popular therapies are nicotine polacrilex ("nicotine gum") and transdermal-delivery systems ("nicotine patch"). These "replacement medications" act to deliver low amounts of nicotine to the user over a period of time to slowly wean the nicotine user off the drug. It is thought that these methods reduce withdrawal symptoms and provide some effects for which the user previously relied on cigarettes (such as desirable mood and attentional states). (Henningfield (1995) *supra.*). These methods, however, suffer from the drawbacks of low penetrance and recidivism of the non-motivated quitter. Moreover, negative effects have been reported by users of nicotine gum such as mouth irritation, sore jaw muscles, dyspepsia, nausea, hiccups and paresthesia. Reported adverse effects from the nicotine patch include skin reactions (itching or erythema), sleep disturbance, gastrointestinal problems, somnolence, nervousness, dizziness and sweating (Haxby (1995) *Am. J. Health-Syst. Pharm.* 52:265-281).

Experimental diagnostic approaches and therapies for treating drug addiction have been suggested in the literature which have yet to be practiced. For example, vaccination as a therapeutic approach for drug addiction has been described previously in principle. Bonese et al. investigated changes in heroin

self-administration by a rhesus monkey after immunization against morphine (Bonese et al. (1974) *Nature* 252: 708-710). Bagasra et al. investigated using cocaine-KLH vaccination as a means to prevent addiction (*Immunopharmacol.* (1992) 23:173-179), although no conclusive results are produced and the methods used by Bagasra are in dispute. (Gallacher (1994) *Immunopharm.* 27:79-81). Obviously, if a conjugate is to be effective in a therapeutic regimen, it must be capable of raising antibodies that can recognize free cocaine, heroin or nicotine circulating *in vivo*. Cerny (WO 92/03163) describes a vaccine and immunoserum against drugs. The vaccine is comprised of a hapten bonded to a carrier protein to produce antibodies. Also disclosed is the production of antibodies against drugs, and the use of these antibodies in the detoxification of one who has taken the drug. *Carrera et al.*, Nature 378:727-730 (1995) discloses the synthesis of a cocaine-KLH vaccine to induce anti-cocaine antibodies which block the locomotor effects of the drug in rats. Blincko, U.S. Patent No. 5,256,409, discloses a vaccine comprising a carrier protein bound to one hapten from the desipramine/imipramine class of drugs and another hapten from the nortriptyline/amitriptyline class of drugs. Liu et al., U.S. Patent No. 5,283,066, discloses use of a hapten-polymeric solid support complex to induce an immune response.

Passive administration of monoclonal antibodies to treat drug abuse has been previously described (*see*, Killian et al. (1978) *Pharmacol. Biochem. Behavior* 9:347-352; Pentel et al. (1991) *Drug Met. Dispositions* 19:24-28). In this approach, pre-formed antibodies to selected drugs are passively administered to animals. While these data provide a demonstration of the feasibility of immunological approaches to addiction therapy, passive immunization as a long term human therapeutic strategy suffers from a number of major drawbacks. First, if antibodies to be used for passive therapy are from non-human sources or are monoclonal antibodies, these preparations will be seen as foreign proteins by the patient, and there may be a rapid immune response to the foreign antibodies. This immune response may neutralize the passively administered antibody, blocking its effectiveness and drastically reducing the time of subsequent

protection. In addition, readministration of the same antibody may become problematic, due to the potential induction of a hypersensitivity response. These problems can be overcome by production of immune immunoglobulin in human donors immunized with the vaccine. This approach is discussed in more detail in the Examples. Second, passively administered antibodies are cleared relatively rapidly from the circulation. The half life of a given antibody *in vivo* is between 2.5 and 23 days, depending on the isotype. Thus, when the antibodies are passively administered, rather than induced by immunization, only short term effectiveness can be achieved.

Another immunological approach to drug addiction has been to use a catalytic antibody which is capable of aiding hydrolysis of the cocaine molecule within the patient (Landry et al. (1993) *Science* **259**:1899-1901). The catalytic antibody is generated by immunization of an experimental animal with a transition state analog of cocaine linked to a carrier protein; a monoclonal antibody is then selected that has the desired catalytic activity. Although this approach is attractive theoretically, it also suffers from some serious problems. Catalytic antibodies must be administered passively and thus suffer from all of the drawbacks of passive antibody therapy. Active immunization to generate a catalytic antibody is not feasible, because enzymatic activity is rare among antibodies raised against transition state analogs, and activity does not appear to be detectable in polyclonal preparations. In addition, the general esterase-like activity of such catalytic antibodies and the uncontrolled nature of the active immune response in genetically diverse individuals makes them potentially toxic molecules, particularly when they are being produced within a human patient.

Yugawa et al. (EP 0 613 899 A2) suggest the use of cocaine-protein conjugate containing a cocaine derivative for raising antibodies for the detection of cocaine or cocaine derivatives in a blood sample. The Syva patents (U.S. Patent No. 3,888,866, No. 4,123,431 and No. 4,197,237) describe conjugates to raise cocaine antibodies for immunoassays. Disclosed are conjugates to BSA using diazonium salts derived from benzoyl ecgonine and cocaine. Conjugates are made using

para-imino ester derivatives of cocaine and norcocaine to conjugate a carrier. Biosite (WO 93/12111) discloses conjugates of cocaine using the paraposition of the phenyl ring of various cocaine derivatives increasing stability to hydrolysis by introducing an amide bond. The Strahilevitz patents (U.S. Patent No. 4,620,977; U.S. Patent 4,813,924; U.S. Patent 4,834,973; and U.S. Patent 5,037,645) disclose using protein conjugates of endogenous substances and drugs for treatment of diseases, preventing dependence on psychoactive haptens, as well as for use in immunoassays, immunodialysis and immunoadsorption.

Bjerke et al. (1987) *Journal of Immunological Methods* 96:239-246 describes the use of a conjugate of cotinine 4'- carboxylic acid bound covalently to poly-L-lysine to generate antibodies to the nicotine metabolite cotinine for use in determining the presence of cotinine in physiological fluids. Additionally, Abad et al. (1993) Anal. Chem. 65(22):3227-3231 describe the use of 3'-(hydroxymethyl) nicotine hemisuccinate conjugated to BSA to generate antibodies to nicotine for use in an ELISA used to measure nicotine in smoke condensates of cigarettes. Neither reference, however, teaches or suggests the use of a nicotine-carrier conjugate for use as a vaccine against nicotine abuse.

No effective therapy for drug addiction, especially, cocaine, heroin and nicotine addiction, has been developed. Thus, there is a need to develop a long term treatment approach to drug addiction, in particular cocaine and nicotine addiction, which does not depend totally on the addicted individual for compliance and self-administration.

SUMMARY OF THE INVENTION

The present invention overcomes the above mentioned drawbacks and provides methods for treating drug abuse. Using therapeutic compositions, in particular hapten-carrier conjugates, the present invention elicits an immune response in the form of anti-drug antibodies within the addict which upon subsequent exposure to the drug in a vaccinated individual neutralizes the drug so the expected pharmacological effects are diminished, if not eliminated. The present invention provides a therapeutic for drug addiction, particularly cocaine, heroin and nicotine addiction, based on vaccination of subjects with a drug/hapten-carrier conjugate, and more particularly, a cocaine-protein, heroin-protein or nicotine-protein conjugate. Therapeutic compositions of the invention comprise at least one hapten and at least one T cell epitope-containing carrier which when conjugated to form a hapten-carrier conjugate is capable of stimulating the production of anti-hapten antibodies. The hapten can be a drug or drug derivative, particularly cocaine, heroin or nicotine. When the therapeutic composition containing the drug/hapten-carrier conjugate is administered to an addicted individual, anti-drug antibodies specific to the drug are elicited. A therapeutic immunization regimen elicits and maintains sufficiently high titers of anti-drug antibodies, such that upon each subsequent exposure to the drug during the period of protection provided by the therapeutic, anti-drug antibodies neutralize a sufficient amount of the drug in order to diminish, if not eliminate, the pharmacological effect of the drug. Also provided are novel methods of preparing these conjugates. A method of passive immunization is also provided, wherein a subject is treated with antibodies generated in a donor by vaccination with the hapten-carrier conjugate of the invention.

These and other features, aspects and advantages of the present invention will become more apparent and better understood with regard to the following drawings, description, and appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1a is a representation of a number of possible, arbitrarily labelled, "branches" of a hapten-carrier conjugate identified for ease of understanding suitable compounds and conjugates used in the practice of the instant invention.

Figure 1b is a representation of a number of possible, arbitrarily labelled, "branches" of a hapten-carrier conjugate identified for ease of understanding suitable compounds and conjugates used in the practice of the instant invention, wherein Q' is a modified T-cell epitope containing carrier, such as a modified protein carrier.

Figure 2 is a representation of the structures of five reagents useful in the practice of the instant invention.

Figure 3 is a representation of the structures of four alternative drugs of abuse suitable for conjugation and administration in accordance with the teachings of the instant invention.

Figure 4a is a representation of a gel showing the relative molecular weights of native (monomer and pentamer) and recombinant cholera toxin-B (CTB) (monomer).

Figure 4b is a representation of a gel illustrating the stability of CTB pentamers over a pH range of 3 - 9.

Figure 4c is a drawing of a Western Blot gel showing 35 peak fractions rCTB#32 and rCTB#53 which were obtained by periplasmic expression resulting in pentameric CTB.

Figure 5a is a graph representing an ELISA where the anti-CTB antibody detects the ability of rCTB to bind to ganglioside GMl on the ELISA plate.

Figure 5b is a scan depicting a flow cytometry binding assay in which rCTB is bound to eukaryotic cells expressing ganglioside GM1.

Figure 6a is a schematic representation of the structural formula of nicotine.

Figure 6b is a diagram representing sites of variability when preparing a nicotine conjugate of the instant invention. The sites of variability are arbitrarily assigned to easily designate the compound and conjugates of the instant invention and not necessarily reaction sites. These sites of variability are as referred to in Figure 7.

Figure 7 is a representation of "branches" at the sites of variability off the nicotine molecule for nicotine conjugates and intermediates of the instant invention. Nicotine conjugates of the present invention are represented when Q is a T cell epitope containing carrier.

Figure 8 is a representation of nicotine metabolites useful in preparation of some of the conjugates of the present invention.

Figures 9a-b shows results of testing of mouse sera in an ELISA for antibody binding to a conjugate of PS-55 and hen egg lysozyme protein (HEL). In Figure 9a, the mice had been immunized with a nicotine-BSA conjugate. In Figure 9b, the mice had been immunized with a nicotine-CTB conjugate.

Figure 10 shows the results of testing anti-nicotine antisera in a competition ELISA for specificity of the antisera for free nicotine when varying concentrations of free nicotine and the nicotine metabolites, cotitine and nornicotine were added. The antisera was prepared in mice by injection of the nicotine conjugate PS-55 BSA. There was little or no recognition of the nicotine metabolites, demonstrating that the anti-nicotine antibodies within the antisera were specific for nicotine. The anesthetic, lidocaine, was used as a negative control and was not able to compete for the binding of antibody. The nicotine conjugate, PS-55 HEL was used as a positive control and its binding to antibody was inhibited by free nicotine.

Figure 11a shows a schematic diagram of the structural formula of heroin

Figure 11b is a diagram representing sites of variability when preparing a heroin conjugate of the instant invention. The sites of variability are arbitrarily assigned to easily designate the compound and conjugates of the instant invention and not necessarily reaction sites. These sites of variability are as referred to in Figure 12.

Figure 12 is a representation of "branches" at the sites of variability off the heroin molecule for heroin conjugates and intermediates of the present invention. Heroin conjugates of the present invention are represented when Q is a T-cell epitope containing carrier.

DETAILED DESCRIPTION OF THE INVENTION

The patent and scientific literature referred to herein establishes the knowledge that is available to those skilled in the art. The issued U.S. Patents, PCT publications, and other publications cited herein are hereby incorporated by reference.

The present invention provides a therapeutic for drug addiction, based on vaccination of an addicted individual with a drug/hapten-carrier conjugate, and more particularly, a heroin-protein conjugate or a nicotine-protein conjugate. Therapeutic compositions of the invention comprise at least one hapten and at least one T cell epitope containing carrier which when conjugated to form a hapten-carrier conjugate is capable of stimulating the production of antihapten antibodies. As used herein the term "T cell epitope" refers to the basic element or smallest unit of recognition by a T cell receptor, where the epitope comprises amino acids essential to receptor recognition. Amino acid sequences which mimic those of the T cell epitopes and which modify the allergic response to protein allergens are within the scope of this invention. A "peptidomimetic" can be defined as chemical structures derived from bioactive peptides which imitate natural molecules. The hapten can be a drug such as heroin, nicotine or drug derivative.

When the therapeutic composition containing the hapten/drug (or derivative thereof) is administered to the addicted individual, anti-drug antibodies specific to the drug are elicited. A therapeutic immunization regimen elicits and maintains sufficiently high titers of anti-drug antibodies, such that upon subsequent exposure to the drug, neutralizing antibodies attach to a sufficient amount of the drug in order to diminish, if not eliminate, the pharmacological effects of the drug. For example, when the therapeutic composition is a heroin-carrier conjugate, treatment induces an anti-heroin antibody response which is capable of reducing or neutralizing heroin in the bloodstream or mucosal tissue of a subject, thereby blocking the psychologically addictive properties of the

drug. Since in the present invention delayed or reduced levels of the drug of abuse reach the central nervous system, the addict receives diminished or no gratification from the use of heroin. This same mechanism of action, when administering a nicotine-carrier conjugate, will induce anti-nicotine antibodies and diminish or extinguish the gratification from the use of nicotine. No side effects are expected from the administration of the therapeutic of the instant invention. For example, the instant drugs-of-abuse are small and monovalent and so are not able to cross-link antibody. Therefore, formation of immune complexes and the associated pathologies are not expected to occur after exposure to the drug of abuse. It is now, and is expected to be, compatible with current and future pharmacological therapies. Further, effective neutralization is long lasting. For example, neutralizing antibody responses against pathogens are known to last for years. Accordingly, it is expected that high-titer anti-drug antibodies elicited using the therapeutic composition of the instant invention can be maintained for long periods of time and possibly, at least a year. This long-term effect of the therapeutic composition with reduced compliance issues reduces recidivism which is a problem with current therapies.

Additionally, the therapeutic vaccination approach of the present invention to heroin addiction is compatible with other therapies currently in use or in clinical trials. In fact, early phase co-therapy is highly desirable because of the time necessary to achieve optimal antibody titers.

Similarly, the therapeutic vaccination approach of the present invention to nicotine addiction is compatible with other therapies for minimizing symptoms of nicotine withdrawal. For example, the nicotine-carrier conjugate of the present invention may be used in conjunction with clonidine, buspirone, and/or antidepressants or sedatives. The vaccine produced by this approach will be compatible with the current nicotine replacement therapies, *i.e.*, gums and patches. Since anti-nicotine antibodies would take several weeks to be generated, some level of craving control would be provided by the use of nicotine replacement therapies.

The following are terms used herein, the definitions of which are provided for guidance. As used herein a "hapten" is a low-molecular-weight organic compound that reacts specifically with an antibody and which is incapable of inciting an immune response by itself but is immunogenic when complexed to a T cell epitope-containing carrier forming a hapten-carrier conjugate. Further, the hapten is characterized as the specificity-determining portion of the hapten-carrier conjugate, that is, it is capable of reacting with an antibody specific to the hapten in its free state. In a non-immunized addicted subject, there is an absence of formation of antibodies to the hapten. The therapeutic composition is used to vaccinate individuals who seek treatment for addiction to drugs. In the instant invention, the term hapten shall include the concept of a more specific drug/hapten which is a drug, an analog of a portion of the drug, or drug derivative. The therapeutic composition, or therapeutic anti-drug vaccine, when initially administered will give rise to a "desired measurable outcome". Initially, the desired measurable outcome is the production of a high titer of anti-drug antibodies. Titer is defined as the serum dilution required for half maximum antibody detection by ELISA. However, manipulation of the dosage regimen suitable for the individual gives and maintains a sustained desired therapeutic effect. The "desired therapeutic effect" is the neutralization of a sufficient fraction of free drug of abuse to reduce or eliminate the pharmacological effects of the drug within a therapeutically acceptable time frame by anti-drug antibodies specific for the drug upon a subsequent exposure to the drug. Determining the therapeutically acceptable time frames for how long it takes to get a sufficient antibody response to a given drug and how long that antibody response is maintained thereto are achieved by those skilled in the art by assessing the characteristics of the subject to be immunized, drug of abuse to be neutralized, as well as the mode of administration. Using this and other vaccination protocols as a model, one skilled in that art would expect the immunity or the period of protection to last several months, up to more than one year.

"Passive immunization" is also disclosed which encompasses administration of or exposure to intact anti-drug antibody or polyclonal antibody or monoclonal antibody fragment (such as Fab, Fv, (Fab')2 or Fab') prepared using the novel conjugates of the instant invention. As stated above, passive immunization of humans with an anti-heroin or anti-nicotine antibody of the present invention as a stand-alone treatment may be less useful than active immunization. Passive immunization would be particularly useful as an initial co-treatment and/or a supplementary complementary treatment (for example, during the period of time after initial administration of the vaccine but before the body's own production of antibodies) or in acute situations to prevent death (for example, when a person presents with a drug overdose). In some situations, passive therapy alone may be preferable, such as when the patient is immunocompromised or needs a rapid treatment.

The drug-conjugates of the present invention, as well as the compositions of the present invention, may also be used as a prophylactic. That is, the drug-conjugates or compositions may be administered to a mammal prior to any exposure to the drug to generate anti-drug antibodies. The generated anti-drug antibodies would be present in the mammal to bind to any drug introduced subsequent to the administration of the conjugate or composition, and therefore minimize or prevent the chance of becoming addicted to the drug.

The therapeutic composition of the instant invention, and more specifically, the therapeutic anti-drug vaccine, is a composition containing at least one drug/hapten-carrier conjugate capable of eliciting the production of a sufficiently high titer of antibodies specific to the drug/hapten such that upon subsequent challenge with the drug/hapten said antibodies are capable of reducing the addictive properties of the drug. The expected immune response to a hapten-carrier conjugate is the formation of both anti-hapten and anti-carrier antibodies. The therapeutic level is reached when a sufficient amount of the anti-drug specific antibodies are elicited and maintained to mount a neutralizing attack on drug introduced after vaccination. The therapeutic regimens of the

instant invention allow for sufficient time for production of antibodies after initial vaccination and any boosting. Further, the optimal antidrug vaccine contains at least one drug/hapten carrier conjugate comprising an optimal combination of the drug as hapten and a carrier so that production of anti-drug antibodies is capable of achieving an optimal therapeutic level, that is, remaining *in vivo* at a sufficiently high titer to withstand a subsequent challenge within several months with the selected drug. More particularly, the antibody titers remain sufficiently high to provide an effective response upon subsequent exposure to the drug for about two months to about one year or more depending upon the individual, more usually at least three months. This optimal composition consists of a hapten-carrier conjugate, excipients and, optionally adjuvants.

When used in the treatment of heroin addiction, the present invention defines a hapten-carrier conjugate, wherein the hapten is heroin or a heroin derivative, which can be used to immunize mammals, particularly humans, to elicit anti-heroin antibodies capable of binding free drug and preventing transit of the drug to the reward system in the brain, thereby abrogating the addictive drug-taking behavior. As discussed in relation to both cocaine and nicotine, anti-heroin antibodies would presumably limit the distribution of heroin across the blood-brain barrier, thus reducing its pharmacological effects

When used in the treatment of nicotine, the present invention defines a hapten-carrier conjugate, wherein the hapten is nicotine or a nicotine derivative, which can be used to immunize mammals, particularly humans, to elicit anti-nicotine antibodies capable of binding free drug and preventing transit of the drug to the reward system in the brain thereby abrogating addictive drug-taking behavior (e.g., smoking cigarettes). It is believed that nicotine binds to the α-subunit of the nicotinic acetylcholine receptors in the brain which results in an increase in dopamine release. It is thought that increased numbers of nicotinic acetylcholine receptors in the brain enhance the physiological dependence of nicotine. As discussed above in relation to heroin, anti-nicotine antibodies would

presumably limit the distribution of nicotine across the blood-brain barrier to the brain, thus reducing its pharmacological effects.

For example, there is some level of standardization with nicotine delivery; that is, each cigarette contains on average 9mg of nicotine of which 1-3mg are effectively dispensed during smoking. Additionally, the peak plasma concentration of nicotine is 25-50ng/ml which is significantly lower than that of cocaine (0.3-1μg/ml). This should provide an ideal opportunity for intervention with moderately high affinity antibodies.

Initial vaccination with the therapeutic hapten-carrier conjugate composition of the present invention creates high titers of hapten-specific antibodies *in vivo*. Periodic tests of the vaccinated subjects' plasma are useful to determine individual effective doses. Titer levels are increased and maintained through periodic boosting. It is anticipated that this therapeutic will be used in combination with current drug rehabilitation programs, including counseling. Further, the therapeutic compositions of the present invention may be aimed at a single drug or several drugs simultaneously or in succession and may be used in combination with other therapies. For example, the therapeutic hapten-carrier conjugate compositions and methods of the instant invention are used without adverse interactions in combination with conventional pharmacological approaches and previously discussed "short term" passive immunization to enhance the overall effect of therapy.

The therapeutic hapten-carrier conjugate composition of the present invention is prepared by coupling one or more hapten molecules to a T cell epitope containing carrier to obtain a hapten-carrier conjugate capable of stimulating T cells (immunogenic) which leads to T cell proliferation and a characteristic release of mediators which activate relevant B cells and stimulate specific antibody production. Antibodies of interest are those specific to the hapten portion of the hapten-carrier conjugate (also called the hapten-carrier complex). Therapeutic compositions containing a combination of conjugates,

either to the same drug (cross-immunization) or to multiple drugs (co-immunization) are disclosed. Such co-mixtures of conjugates of multiple drugs are particularly useful in the treatment of polydrug abuse.

In selecting a drug suitable for conjugation according to the instant invention, one skilled in the art would select a drug with properties likely to elicit high antibody titers. However, if the chosen molecule is similar to those molecules which are endogenous to the individual, antibodies raised against such a molecule could cross-react with many different molecules in the body giving an undesired effect. Thus, the drug to be selected as the hapten (drug/hapten) must be sufficiently foreign and of a sufficient size so as to avoid eliciting antibodies to molecules commonly found inside a human body. For these reasons, alcohol, for example, would not be suitable for the therapeutic of the instant invention. The antibodies raised against the therapeutic composition are highly specific and of a sufficient quantity to neutralize the drug either in the blood stream or in the mucosa or both. Without limiting the invention, the drugs which are suitable for therapeutic composition (not in order of importance) are:

Hallucinogens, for example mescaline and LSD;

Cannabinoids, for example THC;

Stimulants, for example amphetamines, cocaine, phenmetrazine, methylphenidate;

Nicotine;

Depressants, for example, nonbarbiturates (e.g. bromides, chloral hydrate etc.), methaqualone, barbiturates, diazepam, flurazepam, phencyclidine, and fluoxetine;

Opium and its derivatives, for example, heroin, methadone, morphine, meperidine, codeine, pentazocine, and propoxyphene; and

"Designer drugs" such as "ecstasy".

Figure 3 shows the structure of four drugs suitable for conjugation according to the instant invention.

The carrier of the instant invention is a molecule containing at least one T cell epitope which is capable of stimulating the T cells of the subject, which in turn help the B cells initiate and maintain sustained antibody production to portions of the entire conjugate, including the hapten portion. Thus, since a carrier is selected because it is immunogenic, a strong immune response to the vaccine in a diverse patient population is expected. The carrier, like the hapten, must be sufficiently foreign to elicit a strong immune response to the vaccine. A conservative, but not essential, approach is to use a carrier to which most patients have not been exposed to avoid the phenomenon of carrier-induced epitope suppression. However, even if carrier-induced epitope suppression does occur, it is manageable as it has been overcome by dose changes (Dijohn et al. (1989) *Lancet* 1415-1418) and other protocol changes (Etlinger et al. (1990) *Science* **249**:423-425), including the use of CTB (Stok et al. (1994) *Vaccine* **12**:521-526). Vaccines which utilize carrier proteins to which patients are already immune are commercially available. Still further, carriers containing a large number of lysines are particularly suitable for conjugation according to the methods of the instant invention. Suitable carrier molecules are numerous and include, but are not limited to:

Bacterial toxins or products , for example, cholera toxin B-(CTB), diphtheria toxin, tetanus toxoid, and pertussis toxin and filamentous hemagglutinin, shiga toxin, pseudomonas exotoxin;

Lectins, for example, ricin-B subunit, abrin and sweet pea lectin;

Sub virals, for example, retrovirus nucleoprotein (retro NP), rabies ribonucleoprotein (rabies RNP), plant viruses (e.g. TMV, cow pea and cauliflower mosaic viruses), vesicular stomatitis virus-nucleocapsid protein (VSV-N), poxvirus vectors and Semliki forest virus vectors; Artificial vehicles, for example, multiantigenic peptides (MAP), microspheres;

Yeast virus-like particles (VLPs);

Malarial protein antigen;

and others such as proteins and peptides as well as any modifications, derivatives or analogs of the above.

To determine features of suitable carriers, initial experiments were performed using bovine serum albumin as a protein carrier. The protein has been ideal for animal experiments, as it is inexpensive and contains large numbers of lysines for conjugation. However, it is less appropriate for human vaccination because the generation of anti-BSA antibodies has the potential to cause adverse responses. Thus, using the results of these experiments, the above-described criteria were applied to a large number of candidate carriers. The result is the list of carriers described above suitable for the practice of the instant invention.

The carrier of a preferred embodiment is a protein or a branched peptide (e.g., multi-antigenic peptides (MAP)) or single chain peptide. An ideal carrier is a protein or peptide which is not commonly used in vaccination in the country in which the therapy is used, thereby avoiding the potential of "carrier induced epitopic suppression." For example, in the U.S., where standard childhood immunization includes diphtheria and tetanus, proteins such as tetanus toxoid and diphtheria toxoid, if unmodified, may be less desirable as appropriate carriers. Further, the carrier protein should not be a protein to which one is tolerant. In humans, this would exclude unmodified human serum albumin. Further, many food proteins would have to be carefully screened before use as a carrier. Again, in humans, bovine serum albumin would be less desirable as a carrier due to the beef in the diet of most humans. Still further, it is highly advantageous if the carrier has inherent immunogenicity/adjuvanticity. A delicate balance must be struck between the desire for immunogenicity of the carrier and the desire to maximize the anti-hapten antibody. Still further, the preferred carrier would be capable of both systemic response and response at the site of exposure. This is particularly true of heroin, cocaine and nicotine which are more frequently

administered across mucosal membranes. The speed of response is especially critical where cocaine or heroin has been smoked. Accordingly, in the case of cocaine, heroin and nicotine, a preferred carrier elicits not only a systemic response but also a pre-existing mucosal antibody response. In such a mucosal response the reaction of antibodies with cocaine, heroin and/or nicotine would happen rapidly enough to counteract the drug before it begins circulating in the blood stream.

One such preferred carrier is cholera toxin B (CTB), a highly immunogenic protein subunit capable of stimulating strong systemic and mucosal antibody responses (Lycke (1992) *J. Immunol.* **150**:4810-4821; Holmgren et al. (1994) *Am. J. Trop. Med. Hyg.* **50**:42-54; Silbart et al. (1988) *J. Immun. Meth.* **109**:103-112; Katz et al. (1993) *Infection Immun.* **61**:1964-1971). This combined IgA and IgG anti-hapten response is highly desirable in blocking heroin or cocaine that is administered nasally or by inhalation, and in blocking nicotine that is absorbed in the mouth and lungs. In addition, CTB has already been shown to be safe for human use in clinical trials for cholera vaccines (Holmgren et al., *supra;* Jertborn et al. (1994) *Vaccine* **12**:1078-1082; "The Jordan Report, Accelerated Development of Vaccines" 1993., *NIAID*, 1993).

Other useful carriers include those with the ability to enhance a mucosal response, more particularly, LTB family of bacterial toxins, retrovirus nucleoprotein (retro NP), rabies ribonucleoprotein (rabies RNP), vesicular stomatitis virus-nucleocapsid protein (VSV-N), and recombinant pox virus subunits.

In yet another embodiment, various proteins derivatives, peptides fragments or analogs, of allergens are used are carriers. These carriers are chosen because they elicit a T cell response capable of providing help for B cell initiation of anti-hapten antibodies. Examples of and methods of making allergen proteins and peptides and their sequences are disclosed in WO 95/27786 published October 19, 1995. An allergen which is particularly suitable as a carrier is

Cryptomeria japonica, more particularly, recombinant *Cry j 1*, the sequence of which has been published with slight variation. In countries other than Japan, *Cryptomeria japonica* is not prevalent. Therefore, Cry *j 1* allergen generally fits one of the criteria of a suitable carrier, that is a carrier to which a subject has not been previously exposed.

Using the methods and compositions of the present invention, and more particularly, the techniques set out in the Examples below, one skilled in the art links the selected drug/hapten with the selected carrier to make the hapten-carrier conjugate of the instant invention.

In one embodiment of the present invention, the antibodies induced by the therapeutic composition act within the time it takes for the drug to travel from the lungs through the heart to the brain. The ability to elicit this antibody response requires the careful selection of the carrier molecule.

Production of Recombinant B Subunit of Cholera Toxin

Cholera toxin is the enterotoxin produced by *Vibrio cholerae* and consists of five identical B subunits with each subunit having a molecular weight of 11.6 KDa (103 amino acids) and one A subunit of 27.2 KDa (230 amino acids) (Finkelstein (1988) *immunochem. Mol. Gen. Anal. Bac. Path.* 85-102). The binding subunit, CTB, binds to ganglioside GM1 on the cell surface (Sixma et al. (1991) *Nature* **351**:371-375; Orlandi et al. (1993) J. *Biol. Chem.* **268**:17038-17044). CTA is the enzymatic subunit which enters the cell and catalyzes ADP-ribosylation of a G protein, constitutively activating adenylate cyclase (Finkelstein (1988) *Immunochem. Mol. Gen. Anal. Bac. Path.* pp. 85-102). In the absence of the A subunit, cholera toxin is not toxic.

Others have disclosed the production of high level recombinant expression of CTB pentamers (L'hoir et al. (1990) *Gene* **89**:47-52; Slos et al. (1994) *Protein Exp. Purif.* 5:518-526). While native CTB is commercially available, it is difficult to rule

out contamination with CTA. Therefore, recombinant CTB has been expressed in E. *coli* and as says have been developed for its characterization. The choleragenoid construct was purchased from the American Type Culture Collection (pursuant to U.S. Patent 4,666,837). Recombinant CTB was cloned from the original vector (pRIT10810) into an expression plasmid (pET11d, Novagen) with an extra N-terminal sequence containing a His6 tag and expressed in *E. coli* to the level of 25 mg/liter of culture. The protein was purified over a Ni^{2+} column using standard techniques and analyzed on SDS-PAGE (see Figures 4a, b and c). The recombinant CTB is monomeric in this assay and is larger than the native CTB monomer due to the N-terminal extension.

Pentameric recombinant CTB was produced both with and without the His tag using the cDNA modified by PCR to include the *Pel b* leader sequence. A C-terminal Stop codon was inserted to remove the His tag. Both constructs were expressed in E. *coli* from the pET22b vector (Novagen). The His tagged protein was purified by Ni^{2+} affinity chromatography as above (13 mg/L). The untagged recombinant CTB was purified by ganglioside GM1 column affinity chromatography as described (Tayot et al. (1981) *Eur. J. Biochem.* **113**:249-258). Recombinant CTB pentamer was shown to bind to ganglioside GMl in an ELISA and reacted with pentamer-specific antibodies in Western blots and ELISA. Recombinant CTB is also available from other sources, such as SBL Vaccin AB.

The pentameric structure of CTB may be preferred for binding to ganglioside GM1. The pentamer is stable to SDS as long as the samples are not boiled, permitting pentamerization to be assessed by SDS-PAGE. The gel in Figure 4a demonstrates that the native CTB is a pentamer and is readily distinguishable from the denatured monomeric CTB. Pentamer structure is maintained over a pH range from 4 to 9 (see Figure 4b), which facilitates a variety of conjugation chemistries. The recombinant CTB initially expressed is monomeric. One way to obtain pentameric CTB is by making adjustments to express properly folded pentameric CTB. It has been found that cytoplasmic expression provides a much higher level of monomeric CTB. One skilled in the

art is aware of methods of folding monomeric CTB into pentameric CTB (see, e.g., L'hoir et al. (1990) *Gene* 89:47- 52). An alternative to re-folding monomeric CTB to obtain pentameric CTB is periplasmic expression which resulted in pentameric recombinant CTB able to bind GM1-ganglioside by ELISA. Figure 5a and Figure 5b show the data supporting this finding. One skilled in the art may find several approaches for obtaining pentameric recombinant CTB have been described, including periplasmic expression with a leader (Slos et al., *supra;* Sandez et al. (1989) *Proc. Nat '1. Acad. Sci.* 86:481-485; Lebens et al. (1993) BioTechnol. 11:1574-1578) or post-translational refolding (L'hoir et al., *supra;* Jobling et al. (1991) *Mol. Microbiol.* 5:1755-1767).

Another useful carrier is cholera toxin which provides improved mucosal response over CTB. It has been reported that the enzymatically active A subunit adjuvant enhances activity (Liang et al. (1988) *J. immunol.* 141:1495-1501; Wilson et al. (1993) *Vaccine* 11:113-118; Snider et al. (1994) *J. Immunol.* 153:647).

One aspect of achieving the conjugate of the instant invention involves modifying the hapten, sufficiently to render it capable of being conjugated or joined to a carrier while maintaining enough of the structure so that it is recognized as free state hapten (for example, as free heroin or nicotine). It is essential that a vaccinated individual has antibodies which recognize free hapten (heroin or nicotine). Radioimmunoassay and competition ELISA assay experiments, explained in more detail in the Examples, can measure antibody titers to free hapten. Antibodies of interest are hapten-specific antibodies and, in some embodiments, are heroin-specific antibodies or nicotine-specific antibodies. It should be recognized that principles and methods used to describe the preferred embodiments may be extended from this disclosure to a wide range of hapten-carrier conjugates useful in the treatment of a variety of drug addictions and toxic responses.

Conjugates

Preparation of the novel nicotine-carrier conjugates of the present invention are derived from nicotine and nicotine metabolites. Figure 8 shows a representation of nicotine and some of its metabolites. Preparation of the novel heroin conjugates of the present invention are derived from heroin and heroin metabolites. Figures 11a and 11b show a representation of heroin and some of the sites of variability for the preparation of heroin conjugates.

The length and nature of the hapten-carrier linkage is such that the hapten is displaced a sufficient distance from the carrier domain to allow its optimal recognition by the antibodies initially raised against it. The length of the linker is optimized by varying the number of $-CH_2-$ groups which are strategically placed within a "branch" selected from the group consisting of:

CJ 0	Q
CJ 1	(CH_2)nQ
CJ 1.1	CO_2Q
CJ 1.2	COQ
CJ 2	$OCO(CH_2)_nQ$
CJ 2.1	OCOCH=Q
CJ 2.2	$OCOCH(O)CH_2$
CJ 2.3	$OCO(CH_2)_nCH(O)CH_2$
CJ 3	$CO(CH_2)_nCOQ$
CJ 3.1	$CO(CH_2)_nCNQ$
CJ 4	$OCO(CH_2)_nCOQ$
CJ 4.1	$OCO(CH_2)_nCNQ$

CJ 5 $CH_2OCO(CH_2)_nCOQ$

CJ 5.1 $CH_2OCO(CH_2)_nCNQ$

CJ 6 $CONH(CH_2)_nQ$

CJ 7 $Y(CH_2)_nQ$

CJ 7.1 $CH_2Y(CH_2)_nQ$

CJ 8 $OCOCH(OH)CH_2Q$

CJ 8.1 $OCO(CH_2)_nCH(OH)CH_2Q$

CJ 9 $OCOC_6H_5$

CJ 10 shown on Figure 2b

CJ 11 $YCO(CH_2)_nCOQ$

and shown in Figures 1a and 1b herein. With regard to the above branches, n is an integer preferably selected from about 2 to about 20, more particularly about 2 to about 8, most preferably 3 to 5; Y is preferably is selected from the group consisting of S, 0, and NH; and Q is preferably selected from the group consisting of:

(1) -H

(2) -OH

(3) $-CH_2$

(4) $-CH_3$

(4a) $-OCH_3$

(5) -COOH

(6) halogen

(7) protein or peptide carrier

(8) modified protein or peptide carrier

(9) activated esters, such as 2-nitro-4-sulfophenyl ester and N-oxysuccinimidyl ester

(10) groups reactive towards carriers or modified carriers such as mixed anhydrides, acyl halides, acyl azides, alkyl halides, N-maleimides, imino esters, isocyanate, isothiocyanate; or

(11) another "branch" identified by its "CJ" reference number.

A T cell epitope containing carrier (e.g., a protein or peptide carrier) may be modified by methods known to those skilled in the art to facilitate conjugation to the hapten (e.g., by thiolation). For example with 2-iminothiolane (Traut's reagent) or by succinylation, etc. For simplicity, (CH_2)nQi where Q = H, may be referred to as (CH_3), methyl or Me, however, it is understood that it fits into the motif as identified in the "branches" as shown in Figures 1a and 1b.

Further abbreviations of commercially obtainable compounds used herein include:

BSA = Bovine serum albumin

DCC = Dicyclohexylcarbodiimide

15 DMF = N,N -Dimethylformamide

EDC (or EDAC) = N~Ethyl-N'-(3-(dimethylamino) propyl) carbodiimide hydrochloride

EDTA = Ethylenediamine tetraacetic acid, di sodium salt HATU = 0-(7-Azabenzotriazol-1-yl)-l, 1, 3,3-tetramethyluronium hexafluorophosphate

NMM = N-Methylmorpholine

HBTh = 2- (1H-Benzotriazole-1-yl) -1,1,3, 3-tetramethyluronium hexafluorophosphate

TNTU = 2- (5-Norbornene-2,3-dicarboximido) -1,1,3,3-tetramethyluronium tetrafluoroborate

PyBroP® = Bromo-tris-pyrrolidino-phosphonium hexafluorophosphate

HOBt = N-Hydroxybenzotriazole

Further the IUPAC nomenclature for several named compounds are:

Nicotine 1-Methyl-2- (3-pyridyl) pyrrolidine

Cotinine N-Methyl-2- (3-pyridyl)--5-pyrrolidone

Reactions

In one embodiment, the precursor of the conjugates PS-54 were synthesized by acylating racemic nornicotine with succinic anhydride in methylene chloride in the presence of two equivalents of diisopropylethylamine. The product of this reaction is then coupled to the lysine residue of a carrier protein using HATU to obtain the conjugates PS-54 (see Example 1, method B).

In another embodiment, the precursors of PS-55, PS-56, PS-57 and PS-58 were synthesized by selectively alkylating the pyridine nitrogen in (S)-(-)-nicotine in anhydrous methanol, with ethyl 3- bromobutyrate, 5-bromovaleric acid, 6-bromohexanoic acid or 8- bromooctanoic acid respectively (see Example 2, methods A, B, C, and D). The products of these reactions were conjugated to a carrier protein using HATU to obtain the conjugates PS-55, PS-56, PS-57 and PS-58 (see Example 3, Method A).

TABLE 1

Conjugate	Carrier Protein	Haptens/ Monomer	Conjugation Method
PS-54	BSA	19.5	Example 1, Method B
PS-54	HEL	3.2	Example 1, Method B
PS-55	BSA	33.2	Example 3, Method A
PS-55	HEL	1.09	Example 3, Method A
PS-56	BSA	27	Example 3, Method A
PS-56	HEL	2.2	Example 3, Method A
PS-57	BSA	81	Example 3, Method A
PS-57	HEL	8	Example 3, Method A
PS-58	BSA	66.8	Example 3, Method A
PS-58	HEL	7.4	Example 3, Method A

This is a non-limiting list of conjugates. Other conjugates have been made with greater than one hapten coupled to the T cell epitope-containing carrier. Preferably, 1 to 100 haptens are coupled to the T cell epitope-containing carrier. Most preferably, 1 to 70 haptens are coupled to the T cell epitope containing carrier.

Methods of synthesizing compounds PS-54, PS-55, PS-56, PS-57 and PS-58 are disclosed in the Examples. Following the methods disclosed, e.g., using activating agents under aqueous conditions, one skilled in the art can synthesize any desired compound.

There is a wide range of compounds which have been developed to facilitate cross-linking of proteins/peptides or conjugation of proteins to derivatized molecules, e.g., haptens. These include, but are not limited, to carboxylic acid derived active esters (activated compounds), mixed anhydrides, acyl halides, acyl azides, alkyl halides, N-maleimides, imino esters, isocyanates and isothiocyanates, which are known to those skilled in the art. These are capable of forming a covalent bond with a reactive group of a protein molecule. Depending upon the activating group, the reactive group is the amino group of a lysine residue on a protein molecule or a thiol group in a carrier protein or a modified carrier protein molecule which, when reacted, result in amide, amine, thioether, amidine urea or thiourea bond formation. One skilled in the art may identify further suitable activating groups, for example, in general reference texts such as *Chemistry of Protein Conjugation and Cross-Linking* (Wong (1991) CRC Press, Inc., Boca Raton, FL). Ideally, conjugation is via a lysine side chain amino group. Most reagents react preferentially with lysine. An especially suitable carrier is CTB as it has 9 lysine residues per monomer in its native form. To determine if conjugated pentameric CTB retains its structure and activity, GM1 ganglioside binding can be assessed.

Applicants have expressed and purified amounts of recombinant CTB which, once optimized, are produced in large fermentation batches. Processes for expressing and purifying recombinant protein are know in the art, see for example, USSN 07/807,529. For example, CTB may be purified by affinity chromatography (Tayot et al. (1981) *Eur. J. Biochem.* **113**:249-258), conjugated to heroin or nicotine derivatives, and the conjugate may then be further purified. The purified CTB and the resulting conjugate are analyzed for purity and for maintenance of the pentameric structure of CTB. Techniques include SDS-PAGE, native PAGE, gel filtration chromatography, Western blotting, direct and GM1-capture ELISA, and competition ELISA with biotinylated CTB. Level of haptenation is measured by mass spectrometry, reverse phase HPLC and by analysis of the increase in UV absorbance resulting from the presence of the

hapten. Both the solubility and the stability of the conjugate are optimized in preparation for full-scale formulation. Details of some of these analyses are given in the Examples.

Although the pentameric structure of CTB is a preferred carrier for practice of the present invention, and G_{M1} binding is an effective assay to determine that the pentameric form of CTB is present, the present invention is not limited to the use of the pentameric form of CTB. Other T cell epitope carriers are encompassed in the invention, as well as other forms of CTB (e.g., monomer, dimer, etc.) that may be manipulated for use in the invention. If a carrier other than the pentameric form of CTB is utilized, then one skilled in the art would use an appropriate assay to determine the presence and activity of the required carrier (e.g., the use of G_{M1} binding to determine the presence of the pentameric form of CTB).

In order to vary levels of haptenation, alternative approaches are taken. In one embodiment the carrier is haptenated with a multivalent heroin or nicotine construct. This idea is based on the concept of multiple antigenic peptides (MAP) (Lu et al. *Mol. Immunol.*, 28:623-630 (1991)). In this system, multiple branched lysine residues are exploited to maximize hapten density and valency. The premise of this approach is that the immune response is enhanced if there are multiple copies of the hapten attached to the same peptide or protein molecule. Therefore, a multivalent hapten which needs to be attached to only one or two sites on the carrier CTB pentamer is prepared as set out herein. The core of such a multiple antigenic hapten is a branched polylysine core as suggested by Tam (Lu et al., *supra).* A chemically reactive handle is preserved by inclusion of a protected Cys residue. After heroin or nicotine haptenation of all available amino groups, the sulfhydryl of Cys is unmasked and made available for coupling to the protein with any of several bifunctional sulfhydryl/amino specific cross-linkers (Yoshitake et al. (1979) *Eur. J. Biochem.* **101**:395-399). A number of dendrimeric structures are used as a core.

Adjuvant

Any adjuvant which does not mask the effect of the carrier is considered useful in the heroin and nicotine therapeutic vaccines of the present invention (see, Edelman (1980) *Rev. Infect. Dis.* 2:370-373). Applicants initial experiments aimed at demonstrating the feasibility of a therapeutic vaccine against cocaine addiction used the powerful adjuvant CFA. However, CFA is not preferred in humans. A useful adjuvant currently licensed for use in humans is alum, including aluminum hydroxide (Spectrum Chem. Mtg. Corp., New Brunswick, NJ) or aluminum phosphate (Spectrum). Typically, the vaccine is adsorbed onto the alum, which has very limited solubility. Preliminary data in the murine model suggests that alum is capable of inducing a strong anti-cocaine antibody response, and MF59 (Chiron, Emeryville, CA) or RIBI adjuvant is also suitable.

Effective immunization with CTB as the carrier protein does not require a powerful adjuvant. As shown in the Examples, high titer anti-nicotine antibody responses were induced by immunization with the CTB-nicotine conjugate either using alum as the adjuvant or in the absence of any added adjuvant. For carriers other than CTB one skilled in the art would be capable of determining an appropriate adjuvant, if needed.

Excipients and Auxiliary Agents

Therapeutic compositions may optionally contain one or more pharmaceutically acceptable excipients including, but not limited to, sterile water, salt solutions such as saline, sodium phosphate, sodium chloride, alcohol, gum arabic, vegetable oils, benzyl alcohols, polyethylene glycol, gelatine, mannitol, carbohydrates, magnesium stearate, viscous paraffin, fatty acid esters, hydroxy methyl cellulose, and buffer. Other suitable excipients may be used by those skilled in that art. The therapeutic composition may optionally comprise at least one auxiliary agent, for example, dispersion media, coatings, such as lipids and liposomes, surfactants such as wetting agents and emulsifiers, lubricants, preservatives such as antibacterial agents and antifungal agents, stabilizers and

other agents well known to those skilled in the art. The composition of the present invention may also contain further adjuvants, agents and/or inert pharmacologically acceptable excipients which may be added to enhance the therapeutic properties of the drug or enable alternative modes of administration.

Highly purified hapten-carrier conjugates produced as discussed above may be formulated into therapeutic compositions of the invention suitable for human therapy. If a therapeutic composition of the invention is to be administered by injection (i.e., subcutaneous injection), then it is preferable that the highly purified hapten-carrier conjugate be soluble in aqueous solution at a pharmaceutically acceptable pH (that is, a range of about 4-9) such that the composition is fluid and easy administration exists. It is possible, however, to administer a composition wherein the highly purified hapten-carrier conjugate is in suspension in aqueous solution and such a suspension is within the scope of the present invention. The composition also optionally includes pharmaceutically acceptable excipients, adjuvant and auxiliary agents or supplementary active compounds. Depending upon the mode of administration, optional ingredients would ensure desirable properties of the therapeutic composition, for example, proper fluidity, prevention of action of undesirable microorganisms, enhanced bioavailability or prolonged absorption.

A therapeutic composition of the invention should be sterile, stable under conditions of manufacture, storage, distribution and use, and preserved against the contaminating action of microorganisms such as bacteria and fungi. A preferred means for manufacturing a therapeutic composition of the invention in order to maintain the integrity of the composition is to prepare the formulation of conjugate and pharmaceutically excipient such that the composition may be in the form of a lyophilized powder which is reconstituted in excipients or auxiliary agents, for example sterile water, just prior to use. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying, freeze-drying or spin drying which yields a

powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

The active compounds of this invention can be processed in accordance with conventional methods of galenic pharmacy to produce therapeutic compositions for administration to patients, e.g., mammals including humans. The preferred modes of administration are intranasal, intratracheal, oral, dermal, and/or injection. One particularly suitable combination of modes of administration comprises an initial injection with intranasal boosts.

For parenteral application, particularly suitable are injectable, sterile solutions, preferably oily or aqueous solutions, as well as suspensions, emulsions, or implants, including suppositories. Ampoules are convenient unit dosages. For enteral application, particularly suitable are tablets, dragees, liquids, suspensions, drops, suppositories, or capsules, which may include enteric coating. A syrup, elixir, or the like can be used wherein a sweetened vehicle is employed.

Sustained or directed release compositions can be formulated, e.g., liposomes or those wherein the active compound (conjugate) is protected with differentially degradable coatings, e.g., by microencapsulation, multiple coatings, etc. It is also possible to freeze-dry the new compounds and use the lyophilizates obtained, for example, for the preparation of products for injection.

For topical application, there are employed as nonsprayable forms, viscous to semi-solid or solid forms comprising a carrier compatible with topical application and having a dynamic viscosity preferably greater than water. Suitable formulations include but are not limited to solutions, suspensions, emulsions, creams, ointments etc., which are, if desired, sterilized or mixed with auxiliary agent. For topical application suitable are sprayable aerosol preparations wherein the active compound, preferably in combination with a suitable excipient or auxiliary agent, is packaged in a squeeze bottle or in admixture with a pressurized volatile, normally gaseous propellant.

An antibody raised through the compositions and methods of the instant invention may have a molecular weight ranging from 150 KDa to 1,000 KDa. When the subject is exposed to free heroin or nicotine after vaccination with the optimized conjugate in the therapeutic composition, the free heroin or nicotine is targeted by heroin-specific or nicotine-specific antibody or antibodies. No changes in the form or structure of the drug are necessary for the antibody to recognize the drug *in vivo*. While not intending to limit the present invention, it is believed that upon exposure of the vaccinated individual to heroin or nicotine, the anti-drug antibodies will block the effects of heroin and nicotine. At least three mechanisms are believed to contribute to the blocking activity. First, antibodies are unable to cross the blood-brain barrier. Therefore, it is believed that heroin or nicotine, when bound to the anti-heroin or anti-nicotine antibody, will not cross the blood-brain barrier and will not be able to exert its effect on the opioid receptors and dopamine transporters, respectively. Second, without being limited to any particular theory, it is believed that the antibody prevents the drug from binding to its receptor by simple steric blockade. This mechanism is expected to be operative in blocking some of the non-CNS effects of the drugs (e.g. cardiac toxicity) and in the activity of antibodies against other drugs with non-CNS targets. Third, both heroin and nicotine have relatively short half-lives *in vivo* due to both enzymatic and non-enzymatic degradation, creating inactive metabolites. Heroin and nicotine, in particular, are sufficiently small drugs so that it is very unlikely that they could cross-link antibodies, thus, it is highly unlikely that physiologically significant immune complex formation will occur for either of the drugs.

Still further embodiments of mucosal applications are used in the practice of the present invention. For example, copolymer microspheres are used to induce or enhance a mucosal immune response. These small, biodegradable microspheres encapsulate and protect the conjugate and facilitate uptake by the mucosal immune system. Although they are most widely used for oral immunization, they also have been reported to be effective with intranasal

immunization (Walker (1994) *Vaccine* 12:387-399). Inert polymers such as poly(lactide-co-glycolide) (PLG) of 1-10μm diameter are particularly useful in this regard (Holmgren et al. (1994) *Am. J. Trop. Med. Hyg.* 50:42-54; Serva (1994) *Science* 265:1522-1524)

In addition to the preferred conjugates, cross-immunization with different conjugates is carried out in order to minimize antibody cross-reactivity. Mice are primed with one conjugate, and then boosted at day 14 with a different conjugate coupled to the same carrier. Only the subset of antibody-secreting B cells that recognize both of the heroin or nicotine conjugates are maximally stimulated and expanded. It is believed that because the two conjugates differ in their point of attachment to the drug molecule, the specificity of the recognition increases. Specificity of the induced antisera is then confirmed by competition ELISA.

Still further, therapeutic compositions containing more than one conjugate stimulate polyclonal antibodies thereby enhancing antibody response upon subsequent challenge.

Dose

Neutralizing antibody responses against pathogens are known to last for years, and it should be possible to achieve a high-titer anti-heroin or anti-nicotine antibody response that is maintained for at least a year. Based on values obtained with conventional vaccines, it should be possible to achieve the concentrations of specific antibody required to neutralize heroin or nicotine plasma concentrations. Pharmacokinetic data in mice, data not shown, clearly demonstrates that physiologically relevant neutralizing antibody concentrations can be achieved. Finally, the ability of maternal antibodies to cross the placenta in women addicted to heroin and/or women who smoke, and thus protect the fetus, represents a further desirable effect of therapeutic heroin and/or nicotine vaccination. Optimizing therapy to be effective across a broad population is always challenging yet those skilled in the art use a careful understanding of various factors in determining the appropriate therapeutic dose. Further, antibody responses could

be monitored using specific ELISAs as set out in the Examples and other antibody based as says.

Genetic variation in elimination rates, interactions with other drugs, disease-induced alterations in elimination and distribution, and other factors combine to yield a wide range of response to vaccine levels in patients given the same dose. Clinical indicators assist the titration of some drugs into the desired range, and no chemical determination is a substitute for careful observations of the response to treatment. Because clearance, half-life accumulation, and steady state plasma levels are difficult to predict, the measurement of anti-drug- of-abuse antibody production is useful as a guide to the optimal dose. Each of the conjugates/carriers/adjuvants of the present invention is evaluated for the ability to induce an antibody response that is best able to bind free heroin or free nicotine in the circulation.

Further details about the effects of carriers and adjuvants on the induction of an antibody response are given in the Examples. Thus, it will be appreciated that the actual preferred amounts of active compound in a specific case will vary according to the specific conjugate being utilized, the particular compositions formulated, the mode of application, and the particular sites and organism being treated. For example, in one embodiment, the therapeutic composition containing a suitable carrier, is given first parenterally and boosted mucosally. As is discussed in more detail herein, this type of immunization with the optimal hapten and carrier combination is very effective in generating primarily IgG systemically and primarily IgA locally.

As set out in the Examples murine models have been used to demonstrate and measure different characteristics of the antibody response, including antibody titer, ability to recognize free nicotine, nicotine binding capacity, affinity for nicotine, specificity of the antibody response, antibody isotype, antibody tissue localization, and the physiological effects of the antibody following nicotine administration.

Antibody Titer

The first screen for vaccination is whether the conjugate of interest induces a high titer antibody response. Antibody titers can be determined using an ELISA assay as is well known in the art. For example, plates are coated with a nicotine-HEL conjugate, washed extensively, and incubated with varying dilutions of the test serum. The plates are again washed and developed with an enzyme-labelled anti-mouse IgG second antibody. Titers are defined as the reciprocal of the dilution of serum that gives 50% of the maximal response.

Antibody titer depends on both the concentration of antibody and on the antibody affinity. In estimating required antibody titer, both the concentration and the affinity of the antibodies are considered by those skilled in the art.

Antibody affinity reflects the amount of antibody-drug complex at equilibrium with unbound antibody and unbound drug- of-abuse, thus:

$$Keq = [Ab+drug\ complex]/[Ab] \times [drug]$$

where [Ab] = molar concentration of unoccupied antibody binding sites; [drug] = molar concentration of unbound drug; and [Ab+drug] = molar concentration of antibody-drug complex.

Specificity of Antibody Response

In order to be maximally effective at blocking the activity of an addictive drug, the induced antibodies must have minimal affinity for pharmacologically inactive metabolites of such drug. Binding of antibodies to pharmacologically inactive metabolites of a drug would reduce the potency of the vaccine. The specificity of the antisera for metabolites is determined in a competition ELISA and by radiolabelled immunoassay. Furthermore, the effectiveness of the vaccine is increased if the induced antibodies bind to the pharmacologically active metabolites and derivatives of a drug.

Additionally, interaction of the antibodies raised with other drugs used in addiction therapy and in other medical procedures should be minimized. In particular, cross reaction with drugs commonly prescribed to heroin and poly drug abusers is avoided. The following drugs are useful as co-treatments, buprenorphine, desipramine, naloxone, haloperidol, chlorproazine, mazindol and bromocriptine, as well as others that may become relevant.

Effect on Heroin LD_{50}

Those conjugates and immunization protocols that are most effective at inducing high titer specific antibody responses are further evaluated for their ability to shift the heroin LD_{50}. In such experiments, heroin-immunized and control carrier-immunized mice are injected i.v. with heroin at doses around the previously defined LD_{50}. Ten mice are used at each point, and the data is analyzed using a Cochran-Mantel-Haenzel Chi--squared test.

In addition, a failure time model is used to analyze the time-to-death induced by heroin. The extent to which the anti-heroin antibodies increase both (a) the dose of heroin required for lethality and (b) the time-to-death are measures of efficacy in this model. These provide a rapid and rigorous test of the *in vivo* efficacy of the antibodies.

Observing the Physiological Effect on Humans

A person who seeks medical attention during an episode of heroin abuse might present with shallow respirations, pupillary miosis, bradycardia, a decrease in body temperature and a general absence of responsiveness to external stimulation. At high levels of overdose, the symptoms progress to cyanosis and death. In addition to the blood levels of heroin, all these factors will be assessed and specific criteria will be established when administration of either active immunization with the vaccine or passive administration of antibodies to humans is contemplated.

Without intending to limit the scope of the invention, the composition and methods of this invention will now be described in detail with reference to nicotine and heroin, and specific embodiments.

Many of the following Examples specifically describe nicotine and anti-nicotine antibody. These examples are, however, applicable to heroin. For example, monitoring of the redistribution of heroin (i.e., diminished amount in the brain) is arrived at by injection of immunized mice with the tritium labelled nicotine (available from NEN), followed by decapitation at various time points. The effect of the anti-heroin antibody on heroin metabolism and clearance can be analyzed either by TLC analysis of plasma taken from ^{3}H heroin injected mice or by HPLC.

It is to be understood that the example and embodiments described herein are for purposes of illustration only, and that various modification in light thereof will be suggested to persons skilled in the art. Accordingly, the following non-limiting Examples are offered for guidance in the practice of the instant invention.

EXAMPLE 1

Preparation of Nicotine Conjugate

Method A To a solution of nornicotine (50 mmol) in methylene chloride was added triethylamine (75 mmol), followed by succinic anhydride (100 mmol). The solution was heated at reflux for 18 hours. The reaction mixture was washed sequentially with 10% aqueous hydrochloric acid, saturated sodium bicarbonate solution, brine and water. After drying ($MgSO_4$) and removal of the solvents under reduced pressure, the residue was purified using silica gel flash chromatography to furnish the desired product.

Method B The succinylated nornicotine was then used to synthesize the nicotine conjugate PS-54 (Figure 7). To a solution of succinylated nornicotine (5 μm) in DMF (0.1 ml), diisopropylethylamine (10 μm) was added followed by HATU (5.5 μm). After 10 minutes, the pale yellow solution was added dropwise to a solution of either HEL or BSA (500 μg) in 0.1 M sodium borate buffer at pH 8.8 (0.9 ml) and the mixture stirred for 18 hours at ambient temperature. The pH of the conjugate solution was adjusted to pH 7.0 by careful addition of 0.1 M aqueous hydrochloric acid, followed by purification by dialysis against PBS. The dialysate was filtered through a 0.2 μm filter and the level of haptenation measured by mass spectral analysis or UV absorbance.

Induction of Nicotine-Specific Antibody Responses

To induce an antibody response specific for a small molecule, or hapten, such as nicotine, it was necessary to link it to a T-cell epitope-containing carrier, e.g., a protein carrier. The carrier is recognized by T-cells which are necessary for the initiation and maintenance of antibody production by nicotine-specific B cells. In this example, the carrier used was BSA. A panel of structurally distinct nicotine-BSA conjugates was produced that were linked through different parts of the nicotine molecule with several different types of linkers (Fig. 6b). The set of different conjugates allowed the testing of different alterations and presentations of the nicotine molecule. Since any given nicotine conjugate may induce variable amounts of antibodies which recognize either the free hapten (nicotine), the carrier, or the conjugate only (and do not recognize nicotine itself), screening of the conjugates was performed as in the following example.

Four Balb/c female mice, 2-3 months of age, were immunized intraperitoneally with 50 μg nicotine-BSA conjugate, PS-55-BSA, in complete Freund's adjuvant. These animals have a well designed reproducible response to the antigens under investigation. A second injection of PS-55-BSA was given on day 21 and the mice were bled on day 35. Sera were tested in an ELISA for antibody binding to a conjugate of PS-55 and hen egg lysozyme protein (HEL) and

are shown in Figure 9a. These data demonstrate that this nicotine-BSA conjugate was able to induce strong antibody responses.

A second group of ten Balb/cbyj female mice, 2-3 months of age, were immunized for the preparation of a sera pool of anti-nicotine antisera. In this experiment, the carrier used was CTB. The mice were immunized by intramuscular injection with 10ug PS-55-CTB in alhydrogel and boosted three times with the same. The mice were bled and the serum was processed separately. The sera from each animal was first tested in a direct nicotine ELISA to measure anti-nicotine antibodies and then tested in a competition ELISA to determine whether the induced antibodies were capable of recognizing free nicotine.

The sera from each animal was tested in an anti-nicotine direct ELISA to measure the antibody produced as follows. Immulon 2 96 well ELISA plates were coated overnight at 4°C with 50ul of 10ug/ml of a second conjugate nicotine and HEL, PS-55-HEL, diluted in 1 X PBS. Plates were blocked with 0.5% gelatin in PBS for 1 hour at room temperature. Plates were washed 3 times with 1 x PBS containing 0.05% Tween-20 (PBS-T). Sera samples were added to the plates starting at a 1/300 dilution for the PS-55-HEL coated plates. The sera was diluted by 3 fold dilutions and incubated on the blocked plates for 2 hours at room temperature. Plates were then washed three times with PBS-T. Biotinylated goat anti-mouse IgG (Lot #J194-N855B or C) was diluted 1/10,000 in PBS-T and 100ul was added to each well and incubated at room temperature for 1 hour. Streptavidin-HRP was diluted 1/10,000 and added to the wells for 30 minutes following washing with PBS-T. Plates were washed 3 times with PBS-T and then developed by adding TMB Substrate to each of the wells. The reaction was stopped after 5 minutes with 1M Phosphoric acid. Plates were read using an ELISA reader at O.D. 450 nm. Sera that generated anti-nicotine antibodies in the direct ELISA was then tested in a competition ELISA.

Recognition of Free Nicotine

To determine whether the induced antibodies are capable of recognizing the free nicotine molecule, a competition ELISA was performed. In this assay, free nicotine competes with PS-55 HEL coated to ELISA plates for the binding of antibodies in the sera. If the antibodies that have a high affinity for nicotine comprise most of the antibodies binding to the PS-55 HEL, then low concentrations of nicotine are capable of effectively inhibiting the antibody binding.

For 3 out of 4 mice described above which were injected with PS-55 BSA, antibody binding to PS-55 HEL was inhibited by free nicotine (data not shown). Note that the presence of antibody specific for the conjugate alone would not be expected to interfere with the action of the anti-nicotine antibody. This indicates that antibody is present in each of these sera that recognizes free nicotine. The major metabolite of nicotine, cotinine, was also tested in the competition ELISA and it cannot compete with antibodies in any of the sera except at very high concentrations. To verify that the induced antibodies were capable of recognizing the free nicotine molecule, an RIA was used to measure specific binding to [^{3}H]-nicotine. Immune sera from the above experiment was incubated with [^{3}H] -nicotine and protein-G-conjugated Sepharose beads (Gammabind-G Sepharose, Pharmacia), which bind IgG in the sera samples. The antibody-bound [^{3}H] nicotine was isolated by centrifugation of the beads and was detected by scintillation counting of the beads. Sera from 3 out of the 4 mice bound significantly to free [^{3}H] -nicotine (data not shown). Pre-incubation of these sera with 50-fold excess unlabeled nicotine completely inhibited the binding of the [^{3}H]-nicotine to these antibodies. These data demonstrate that nicotine-carrier conjugates have been synthesized which induce nicotine-specific antibody responses that should be capable of preventing the distribution of nicotine to the brain *in vivo*.

For the second group of 10 mice which were immunized using PS-55-CTB, antibody binding to PS-55 HEL was inhibited by free nicotine (Figure 10). One dilution of sera was used which represented the 50% point titer. Immulon 2 96 well ELISA plates were coated overnight at 4°C with 50ul of 10ug/ml PS-N-3.2 HEL diluted in 1 X PBS. Plates were blocked with 0.5% gelatin in PBS for 1 hour at room temperature. Plates were washed 3 times with 1 x PBS containing 0.05% Tween-20 (PBS-T). The plates were incubated with antiserum in the presence of varying concentrations of free nicotine as well as the metabolites, drugs, and related compounds for 2 hours at room temperature. Plates were then washed three times with PBS-T. Biotinylated goat anti-mouse IgG (Lot #J194-N855B or C) was diluted 1/10,000 in PBS-T and 100ul was added to each well and incubated at room temperature for 1 hour. Streptavidin-HRP was diluted 1/10,000 and added to the wells for 30 minutes following washing with PBS-T. Plates were washed 3 times with PBS-T and then developed by adding TMB Substrate to each of the wells. The reaction was stopped after 5 minutes with 1M Phosphoric acid. Plates were read using an ELISA reader at O.D. 450 nm. The competition curves for each sera pool tested are shown in Figure 10. Increasing concentrations of competitor were graphed out on the x-axis and the absorbance at 450 nm was on the y-axis.

As depicted in Figure 10, there was little or no recognition to the metabolites of nicotine, cotinine or nornicotine. The anesthetic, lidocaine, was used as a negative control and was not able to compete the binding of the antibody. The conjugate itself was also used as a competitor and was able to compete binding of this antibody.

In order to be maximally effective at blocking the activity of nicotine, the induced antibodies should have minimal affinity for the metabolites of nicotine. Binding of the antibodies to the more stable metabolites would reduce the potency of the vaccine. In screening the pool of antisera only nicotine was able to inhibit binding of the antibody to the conjugate. In this experiment, little or no recognition was seen with the metabolites (cotinine and nornicotine), and anesthetic (lidocaine). Therefore, the nicotine-CTB conjugate induces antibodies

that recognize nicotine and do not recognize the less active metabolites. The nicotine-CTB conjugate also does not recognize the compounds which have related structures and the drugs which are currently being used to treat nicotine addiction (data not shown).

Specificity of Nicotine-Specific Antibodies

To analyze the specificity of the anti-nicotine antibodies induced by the nicotine vaccine, sera from the mice immunized with nicotine-CTB conjugate are tested in a competition ELISA. A panel of metabolites of nicotine and related molecules are tested at varying concentrations. If the antibodies have high affinity for the metabolite, then low concentrations are capable of effectively competing this assay. The relative reactivity is expressed as the IC_{50}, the concentration of the inhibitor that decreases the ELISA signal by 50%. The following metabolites are tested for reactivity: nicotine glucuronide, cotinine, cotinine glucuronide, trans-3'-hydroxycotinine, trans-3'-hydroxycotinine glucuronide, nicotine 1'-N-oxide, cotinine N-oxide, and nornicotine.

Efficacy of Nicotine-Specific Antibody in Inhibiting Nicotine Distribution

in vivo Inhibition of Nicotine Distribution to the Brain

To assess changes in nicotine tissue distribution caused by nicotine-specific antibody, ^{3}H-nicotine distribution is followed in nicotine-CTB-immunized mice compared to naive (unimmunized) control mice. Immune and control immunized mice are injected with 0.08 mg/kg ^{3}H-nicotine i.v. and then decapitated at 1.0 minutes after injection. Brains and blood (plasma) are removed for subsequent analysis of tissue and plasma nicotine concentration. Blood is collected into tubes containing EDTA to prevent clotting. Brains and plasma samples are placed into scintillation vials containing tissue stabilizer; digestion of samples occurs over 3 days at room temperature. The samples are decolorized and scintillation cocktail is added to each sample. Glacial acetic acid is added to clarify the samples. After the samples are counted in a scintillation counter, data are converted to ng/g or

ng/ml of tissue. Nicotine concentration in the brain tissue of nicotine-CTB immunized mice is significantly lower after injection of ^{3}H-nicotine than in brain tissue of naive control mice (data not shown).

Example 2

Method A *N' -Butyric acid adduct of (S) -Nicotine*

To a solution of (S) -nicotine (0.031 moles) in anhydrous methanol (50ml) at ice-water temperature under argon, ethyl-4- bromobutyrate (0.0341 moles) was added dropwise over 10 minutes. The resulting orange colored solution was allowed to warm to ambient temperature and stirred for 18 hours. The solvents were removed under reduced pressure leaving a brown residue which was precipitated with hexane to give an analytically pure sample of the desired ester.

The ester (36mg) was dissolved in methanol (3ml) and lM sodium hydroxide solution (5ml) and stirred for 18 hours at ambient temperature. The solvents were removed under reduced pressure and the residue dissolved in 10% hydrochloric acid and extracted with ethyl acetate. Following drying ($MgSO_4$) the solvents were removed under reduced pressure to yield the desired compound.

Method B *N'-Valeric acid adduct of (S) -Nicotine*

To a solution of (S)-nicotine (0.031 moles) in anhydrous methanol (50ml) at ice-water temperature under argon, 1- bromovaleric acid (0.0341 moles) was added dropwise over 10 minutes. The resulting orange colored solution was allowed to warm to ambient temperature and stirred for 18 hours. The solvents were removed under reduced pressure leaving a brown residue which was precipitated with hexane to give an analytically pure sample of the desired compound.

Method C *N'-Hexanoic acid adduct of (S) -Nicotine*

To a solution of (S) -nicotine (0.031 moles) in anhydrous methanol (50ml) at ice-water temperature under argon, 1- bromohexanoic acid (0.0341 moles) was added dropwise over 10 minutes. The resulting orange colored solution was

allowed to warm to ambient temperature and stirred for 18 hours. The solvents were removed under reduced pressure leaving a brown residue which was precipitated with hexane to give an analytically pure sample of the desired compound.

Method D *N'-Octanoic acid adduct of (S) -Nicotine*

To a solution of (S) -nicotine (0.031 moles) in anhydrous methanol (50ml) at ice-water temperature under argon, the appropriate 1-bromooctanoic acid (0.0341 moles) was added dropwise over 10 minutes. The resulting orange colored solution was allowed to warm to ambient temperature and stirred for 18 hours. The solvents were removed under reduced pressure leaving a brown residue which was precipitated with hexane to give an analytically pure sample of the desired compound.

Example 3

Method A *General preparation of PS-55, PS-56, PS-57 and PS-58*

To a solution of the appropriate N'-alkanoic acid analog of nicotine (6.27×10^{-5} moles) (from Example 1) in DMF (1.6ml), DIEA (1.25×10^{-4} moles) and HATU (7.53×10^{-5} moles) were added. After 10 minutes at ambient temperature, the pale yellow solution was added to either HEL or BSA (16.5 mg) in 0.1M sodium bicarbonate, pH 8.3 (14.4ml) and stirred for 18 hours. The conjugate solution was purified by dialysis against PBS at 4°C overnight. The conjugates were analyzed using laser desorption mass spectral analysis to determine the number of haptens.

Method B *Preparation of PS-55*

To a solution of the N'-butyric acid adduct of nicotine (39mg, 1.56×10^{-4} mole) (from Example 2, Method A) in DMF (0.4ml), DIEA (54ml, 3.10×10^{-4} mole) and HATU (71mg, 1.87×10^{-4} mole) were added. After 10 minutes at ambient temperature, the pale yellow solution was added dropwise to rCTB (2mg, 3.4×10^{-8}

moles [$15.6x10^{-6}$ moles of lysines]) in 4ml of 0.1M sodium borate, 0.15M sodium chloride, pH8.5 buffer and then stirred for 1 hour. The conjugate was purified by dialysis against PBS at 4°C overnight. The conjugate was analyzed using laser desorption mass spectral analysis to determine the number of haptens.

Method C *Preparation of PS-56*

To a solution of the N'-valeric adduct of nicotine (2mg) (from Example 2, Method B) in DMF (0.4ml), DIEA (2ml) and HATU (2mg) were added. After 10 minutes at ambient temperature, the pale yellow solution was added dropwise to rCTB (2mg, $3.4x10^{-5}$ moles [$15.6x10^{-6}$ moles of lysines]) in 4ml of 0.1M sodium borate, 0.15M sodium chloride, pH8.5 buffer and then stirred for 1 hour. The conjugate was purified by dialysis against PBS at 4°C overnight. The conjugate was analyzed using laser desorption mass spectral analysis to determine the number of haptens.

Method D *Preparation of PS-57*

To a solution of the N'-hexanoic acid adduct of nicotine (2mg) (from Example 2, Method C) in DMF (0.4ml), DIEA (2ml) and HATU (2mg) were added. After 10 minutes at ambient temperature, the pale yellow solution was added dropwise to rCTB (2mg, $3.4x10^{-8}$ moles [$15.6x10^{-6}$ moles of lysines]) in 4ml of 0.1M sodium borate, 0.15M sodium chloride, pH8.5 buffer and then stirred for 1 hour. The conjugate was purified by dialysis against PBS at 4°C overnight. The conjugate was analyzed using laser desorption mass spectral analysis to determine the number of haptens.

Method E *Preparation of PS-58*

To a solution of the N'-octanoic acid adduct of nicotine (2mg) (from Example 2, Method D) in DMF (0.4ml), DIEA (2ml) and HATU (2mg) were added. After 10 minutes at ambient temperature, the pale yellow solution was added dropwise to rCTB (2mg, $3.4x10^{-8}$ moles [$15.6x10^{-6}$ moles of lysines]) in 4ml of 0.1M sodium borate, 0.15M sodium chloride, pH8.5 buffer and then stirred for 1 hour.

The conjugate was purified by dialysis against PBS at 4°C overnight. The conjugate was analyzed using laser desorption mass spectral analysis to determine the number of haptens.

Method F *Preparation of PS-60*

i) Preparation of the N-pyrrolidine adduct

To a solution nornicotine (10mmol) in methanol, ethyl-5-bromovalerate (10mmol) is added dropwise. After consumption of the starting material, as indicated by TLC, the solvents are removed under reduced pressure and the residue is purified using silica gel flash chromatography to furnish the desired N-pyrrolidine adduct.

ii) Preparation of the conjugate

The ester (15mmol) is dissolved in 5% aqueous methanol and to this is added sodium hydroxide (15mmol). After comsumption of the starting material, as indicated by TLC, the pH is taken to pH2 by careful addition of 1M aqueous hydrochloric acid and then extracted with ethyl acetate. After drying ($MgSO_4$) and removal of the solvents under reduced pressure, the product is purified using silca gel flash chromatography to furnish the desired free acid.

To a solution of the free acid ($1.55x10^{-4}$ mole) in DMF (0.4ml) is added DIEA ($3.1x10^{-4}$ mole) followed by HATU ($1.86x10^{-4}$ mole). After 10 minutes at ambient temperature, the pale yellow solution is added dropwise to rCTB (2mg, $3.4x10^{-8}$ mole [$15.5x10^{-6}$ moles of lysines]) in 4ml of 0.1M sodium borate, 0.15M sodium chloride at pH8.5 buffer. The mixture is kept at ambient temperature for 1 hour, neutralized and then dialyzed extensively against PBS at 4°C to furnish PS-60.

Method G *Preparation of PS-51*

i) Preparation of methyl 6-methylnicotinate

6-Methylnicotinate (0.375mol) is added to a refluxing solution of concentrated sulfuric acid (25ml) in methanol (250ml) and stirred at reflux for 3 hours. Methanol (250ml) is then added and the resultant mixture heated at reflux for an additional 18 hours. The reaction mixture is cooled and concentrated under reduced pressure to a slurry which is added to a solution of sodium bicarbonate (80g) in water (450ml). The mixture is concentrated under reduced pressure to remove most of the methanol. The resultant turbid mixture is extracted with methylene chloride, dried ($MgSO_4$), filtered and concentrated under reduced pressure. The resulting crude product is purified by distillation under reduced pressure to furnish the desired ester.

ii) **Preparation of 6-methylmyosmine**

To a solution of diisopropylamine (0.33mol) in diethyl ether (500ml) under argon at -70°C is added n-butyllithium (0.247mol of a 1.6M solution in hexane). To the prepared lithium diisopropylamine (LDA) is added N-(trimethylsilyl) pyrrolidinone (0.265mol) and the solution stirred for 15 minutes at -70°C. To this solution is added methyl 6-methylnicotinate (0.165mol) in diethyl ether (25ml) and the mixture is allowed to warm to ambient temperature overnight. After this, the mixture is cooled in an ice-bath, water (33ml) is added and the ether layer is decanted off. Additional ether is added and the decanting procedure is repeated twice. To the aqueous layer is added concentrated hydrochloric acid and the resulting solution is refluxed overnight. The acidic solution is washed with diethyl ether, concentrated under reduced pressure, cooled in an ice-bath and basified with 50% aqueous potassium hydroxide. The aqueous layer is extracted with diethyl ether (3 x 150ml), dried (Na_2SO_4), concentrated under reduced pressure and purified by distillation under reduced pressure to furnish the desired 6-methylmyosmine.

iii) **Preparation of ethyl valerate adduct of 6-methylmyosmine**

To a solution of 6-methylmyosmine (10mmol) in dry toluene is added sodium amide (15mmol). After 10 minutes, ethyl-5-bromovalerate (20mmol) is added and

the mixture stirred until the starting material is consumed, as indicated by TLC. The mixture is cooled in an ice-bath, quenched with dry ethanol, extracted with ethyl acetate, dried ($MgSO_4$) and concentrated under reduced pressure. Purification using silica gel flash chromatography furnishes the desired product.

iv) Preparation of valeric acid adduct of 6-methylnornicotine

To a solution of the adduct (10mmol) from (iii) above in methanol is added sodium cyanoborohydride (10mmol), a trace of bromocresol green indicator and enough 2N HCl/methanol such that the color of the solution turns from blue to yellow and remains yellow. The solution is allowed to stir for several hours, after which 6N aqueous hydrochloric acid is added and the mixture concentrated under reduced pressure. After basifying with sodium bicarbonate solution, diethyl ether extraction and drying ($MgSO_4$), the material is purified using distillation under reduced pressure to yield the desired compound.

v) Preparation of valeric acid adduct of 6-methylnicotine

To a solution of the nornicotine (10mmol) adduct from (iv) above in diethyl ether is added iodomethane (20mmol). The solution is refluxed under argon using an efficient condenser until the starting material is consumed, as indicated by TLC. The solvents are removed under reduced pressure and the residue is purified using flash chromatography to produce a racemic mixture of the desired compound. The isomers are separated using chiral HPLC to furnish the desired (S)-isomer.

vi) Preparation of the conjugate

To a solution of the (S)-isomer of the 6-methylnicotine derivative ($1.53x10^{-5}$ moles) from (v) above in DMF is added DIEA ($3.06x10^{-5}$ mole) followed by HATU ($1.86x10^{-5}$ mole). After 10 minutes at ambient temperature the pale yellow solution is added to rCTB (2mg, $3.40x10^{-8}$ moles of protein; $1.53x10^{-6}$ moles of lysines) in 4ml of 0.1M sodium borate, 0.15M NaCl, pH8.5 buffer. After 1 hour at ambient

temperature, the solution is extensively dialyzed against PBS at 4°C and the number of haptens analyzed using mass spectral analysis.

Method H *Preparation of PS-52*

i) Preparation of methyl 5-methlynicotinate

5-Methylnicotinate (0.375mol) is added to a refluxing solution of concentrated sulfuric acid (25ml) in methanol (250ml) and stirred at reflux for 3 hours. Methanol (250ml) is then added and the resultant mixture heated at reflux for an additional 18 hours. The reaction mixture is cooled and concentrated under reduced pressure to a slurry which is added to a solution of sodium bicarbonate (80g) in water (450ml). The mixture is concentrated under reduced pressure to remove most of the methanol. The resultant turbid mixture is extracted with methylene chloride, dried ($MgSO_4$), filtered and concentrated under reduced pressure. The resulting crude product is purified by distillation under reduced pressure to furnish the desired ester.

ii) Preparation of 5-methylmyosmine

To a solution of diisopropylamine (0.33mol) in diethyl ether (500ml) under argon at -70°C is added n-butyllithium (0.247mol of a 1.6M solution in hexane). To the prepared lithium diisopropylamine (LDA) is added N-(trimethylsilyl) pyrrolidinone (0.265mol) and the solution stirred for 15 minutes at -70°C. To this solution is added methyl 6-methylnicotinate (0.165mol) in diethyl ether (25ml) and the mixture is allowed to warm to ambient temperature overnight. After this, the mixture is cooled in an ice-bath, water (33ml) is added and the ether layer is decanted off. Additional ether is added and the decanting procedure is repeated twice. To the aqueous layer is added concentrated hydrochloric acid and the resulting solution is refluxed overnight. The acidic solution is washed with diethyl ether, concentrated under reduced pressure, cooled in an ice-bath and basified with 50% aqueous potassium hydroxide. The aqueous layer is extracted with diethyl ether (3 x 150ml), dried (Na_2SO_4), concentrated under reduced

pressure and purified by distillation under reduced pressure to furnish the desired 5-methylmyosmine.

iii) Preparation of ethyl valerate adduct of 5-methylmyosmine

To a solution of 5-methylmyosmine (10mmol) in dry toluene is added sodium amide (15mmol). After 10 minutes, ethyl-5-bromovalerate (20mmol) is added and the mixture stirred until the starting material is consumed, as indicated by TLC. The mixture is cooled in an ice-bath, quenched with dry ethanol, extracted with ethyl acetate, dried ($MgSO_4$) and concentrated under reduced pressure. Purification using silica gel flash chromatography furnishes the desired product.

iv) Preparation of valeric acid adduct of 5-methylnornicotine

To a solution of the adduct (10mmol) from (iii) above in methanol is added sodium cyanoborohydride (10mmol), a trace of bromocresol green indicator and enough 2N HCl/methanol such that the color of the solution turns from blue to yellow and remains yellow. The solution is allowed to stir for several hours, after which 6N aqueous hydrochloric acid is added and the mixture concentrated under reduced pressure. After basifying with sodium bicarbonate solution, diethyl ether extraction and drying ($MgSO_4$), the material is purified using distillation under reduced pressure to yield the desired compound.

v) Preparation of valeric acid adduct of 5-methylnicotine

To a solution of the nornicotine (10mmol) adduct from (iv) above in diethyl ether is added iodomethane (20mmol). The solution is refluxed under argon using an efficient condenser until the starting material is consumed, as indicated by TLC. The solvents are removed under reduced pressure and the residue is purified using flash chromatography to produce a racemic mixture of the desired compound. The isomers are separated using chiral HPLC to furnish the desired (S)-isomer.

vi) Preparation of the conjugate

To a solution of the (S)-isomer of the 5-methylnicotine derivative ($1.53x10^{-5}$ moles) from (v) above in DMF is added DIEA ($3.06x10^{-5}$ mole)) followed by HATU ($1.86x10^{-5}$ mole). After 10 minutes at ambient temperature, the pale yellow solution is added to rCTB (2mg, $3.40x10^{-8}$ moles of protein; $1.53x10^{-6}$ moles of lysine) in 4ml of 0.1M sodium borate, 0.15M NaCl, pH8.5 buffer. After 1 hour at ambient temperature, the solution is extensively dialyzed against PBS at 4°C and the number of haptens analyzed using mass spectral analysis.

Method I *Preparation of PS-53*

(i) Preparation of methyl 4-methylnicotinate

4-Methylnicotinate (0.375mol) is added to a refluxing solution of concentrated sulfuric acid (25ml) in methanol (250ml) and stirred at reflux for 3 hours. Methanol (250ml) is then added and the resultant mixture heated at reflux for an additional 18 hours. The reaction mixture is cooled and concentrated under reduced pressure to a slurry which is added to a solution of sodium bicarbonate (80g) in water (450ml). The mixture is concentrated under reduced pressure to remove most of the methanol. The resultant turbid mixture is extracted with methylene chloride, dried ($MgSO_4$), filtered and concentrated under reduced pressure. The resulting crude product is purified by distillation under reduced pressure to furnish the desired ester.

(ii) Preparation of 4-methylmyosmine

To a solution of diisopropylamine (0.33mol) in diethyl ether (500ml) under argon at -70°C is added n-butyllithium (0.247mol of a 1.6M solution in hexane). To the prepared lithium diisopropylamine (LDA) is added N-(trimethlsilyl) pyrrolidinone (0.265mol) and the solution stirred for 15 minutes at -70°C. To this solution is added methyl 6-methylnicotinate ((0.165mol) in diethyl ether (25ml) and the mixture is allowed to warm to ambient temperature overnight. After this, the mixture is cooled in an ice-bath, water (33ml) is added and the the layer is decanted off. Additional ether is added and the decanting procedure is repeated

twice. To the aqueous layer is added concentrated hydrochloric acid and the resulting solution is refluxed overnight. The acidic solution is washed with diethyl ether, concentrated under reduced pressure, cooled in an ice-bath and basified with 50% aqueous potassium hydroxide. The aqueous layer is extracted with diethyl ether (3 x 150ml), dried (Na_2SO_4), concentrated under reduced pressure and purified by distillation under reduced pressure to furnish the desired 4-methylmyosmine.

Example 4

Preparation of Heroin Conjugates

Preparation of PS-61

i) ***Preparation of norheroin***

To a solution of heroin (10mmol) in water at 0°C is added potassium permanganate (12mmol). After consumption of the starting material, as indicated by TLC, the suspension is allowed to warm to ambient temperature. The manganese dioxide is then removed by filtration and the solvents removed under reduced pressure to furnish norheroin as the desired product.

ii) Preparation of conjugate precursor

To a solution of the norheroin (10mmol) in THF is added ethyl-5-bromovalerate (20mmol) dropwise. After consumption of the starting material, as indicated by TLC, the solvents are removed under reduced pressure. The residue is then purified on silica gel using flash chromatography to furnish the desired ester adduct of norheroin.

iii) Preparation of the conjugate

The ester (15mmol) is dissolved in 5% aqueous methanol and to this is added sodium hydroxide (15mmol). After comsumption of the starting material, as indicated by TLC, the pH is taken to pH2 by careful addition of 1M aqueous

hydrochloric acid and then extracted with ethyl acetate. After drying ($MgSO_4$) and removal of the solvents under reduced pressure, the product is purified using silca gel flash chromatography to furnish the desired free acid.

To a solution of the free acid ($1.55x10^{-4}$ mole) in DMF (0.4ml) is added DIEA ($3.1x10^{-4}$ mole) followed by HATU ($1.86x10^{-4}$ mole). After 10 minutes at ambient temperature, the pale yellow solution is added dropwise to rCTB (2mg,$3.4x10^{-8}$ mole [$15.5x10^{-6}$ moles of lysines]) in 4ml of 0.1M sodium borate, 0.15M sodium chloride at pH8.5 buffer. The mixture is kept at ambient temperature for 1 hour, neutralized and then dialyzed extensively against PBS at 4°C to furnish PS-61. The conjugate is analyzed using laser desorption mass spectral analysis to determine the number of haptens.

Example 5

Preparation of PS-62 and PS-63

(i) Preparation of precursors

To a solution of heroin (10mmol) in dry THF at 0oC, n-butyllithium (1.5mmol of a 1.6M solution in hexanes) is added dropwise . The resulting mixture is kept at 0oC for 2 hours and then ethyl-4-bromobutyrate (22mmol) in THF is added dropwise over 10 minutes. The resulting mixture is then heated until the starting material is consumed, as indicated by TLC. After this, the reaction mixture is cooled to 0oC and 10% aqueous hydrochloric acid is added carefully. The two layers are separated and the aqueous layer extracted with ethyl acetate. The combined organic extracts are then washed sequentially with 1M aqueous sodium hydroxide solution, water and brine. After drying ($MgSO_4$), the solvents are removed under reduced pressure and the residue is purified using silica gel flash chromatography to furnish two products, one *ortho* and one *meta* to the aromatic acetate group.

(ii) Preparation of PS-62

The ester of the *ortho*adduct (5mmol) is dissolved in 5% aqueous methanol and to this is added sodium hydroxide (5mmol). After comsumption of the starting material, as indicated by TLC, the pH is taken to pH2 by careful addition of 1M aqueous hydrochloric acid and then extracted with ethyl acetate. After drying ($MgSO_4$) and removal of the solvents under reduced pressure, the product is purified using silca gel flash chromatography to furnish the desired free acid.

To a solution of the free acid ($1.55x10^{-4}$ mole) in DMF (0.4ml) is added DIEA ($3.1x10^{-4}$ mole) followed by HATU ($1.86x10^{-4}$ mole). After 10 minutes at ambient temperature, the pale yellow solution is added dropwise to rCTB (2mg,$3.4x10^{-8}$ mole [$15.5x10^{-6}$ moles of lysines]) in 4ml of 0.1M sodium borate, 0.15M sodium chloride at pH8.5 buffer. The mixture is kept at ambient temperature for 1 hour, neutralized and then dialyzed extensively against PBS at 4°C to furnish PS-62. The conjugate is analyzed using laser desorption mass spectral analysis to determine the number of haptens.

(iii) Preparation of PS-63

The ester of the *meta* adduct (5mmol) is dissolved in 5% aqueous methanol and to this is added sodium hydroxide (5mmol). After comsumption of the starting material, as indicated by TLC, the pH is taken to pH2 by careful addition of 1M aqueous hydrochloric acid and then extracted with ethyl acetate. After drying ($MgSO_4$) and removal of the solvents under reduced pressure, the product is purified using silca gel flash chromatography to furnish the desired free acid.

To a solution of the free acid ($1.55x10^{-4}$ mole) in DMF (0.4ml) is added DIEA ($3.1x10^{-4}$ mole) followed by HATU ($1.86x10^{-4}$ mole). After 10 minutes at ambient temperature, the pale yellow solution is added dropwise to rCTB (2mg,$3.4x10^{-8}$ mole [$15.5x10^{-6}$ moles of lysines]) in 4ml of 0.1M sodium borate, 0.15M sodium chloride at pH8.5 buffer. The mixture is kept at ambient temperature for 1 hour, neutralized and then dialyzed extensively against PBS at 4°C to furnish PS-63. The conjugate is analyzed using laser desorption mass spectral analysis to determine the number of haptens.

Example 6

Preparation of PS-64

i) Preparation of acetylated codeine

To a solution of codeine (10mmol) in methylene chloride is added triethylamine (12mmol), followed by acetic anhydride (12mmol). After consumption of the starting material, as indicated by TLC, the solvents are removed under reduced pressure and the residue purified using silica gel flash chromatography to furnish the desired acetylated product.

ii) Demethylation of acetylated codeine

To a solution of the acetylated product (10mmol) in methylene chloride, boron tribromide (12mmol of a 1.0M solution in methylene chloride) was added dropwise. After consumption of the starting material, as indicated by TLC, anhydrous methanol is added carefully and the mixture concentrated under reduced pressure. The residue is dissolved in methanol, re-concentrated under reduced pressure and then purified using silica gel flash chromatography to furnish the desired alcohol.

iii) Succinylation of demethylated acetylated codeine

To a solution of the alcohol (10mmol) in methylene chloride is added triethylamine (20mmol) followed by succinic anhydride (20mmol). The resulting mixture is heated at reflux until the starting matertial is consumed, as indicated by TLC. After this, the solvents are removed under reduced pressure and the residue purified using silica gel flash chromatography to furnish the desired hemisuccinate.

iv) Preparation of PS-64

To a solution of the hemisuccinate (1.55×10^{-4} mole) in DMF (0.4ml) is added DIEA (3.1×10^{-4} mole) followed by HATU (1.86×10^{-4} mole). After 10 minutes at

ambient temperature, the pale yellow solution is added dropwise to rCTB (2mg,$3.4x10^{-8}$ mole [$15.5x10^{-6}$ moles of lysines]) in 4ml of 0.1M sodium borate, 0.15M sodium chloride at pH8.5 buffer. The mixture is kept at ambient temperature for 1 hour, neutralized and then dialyzed extensively against PBS at 4°C to furnish PS-64. The conjugate is analyzed using laser desorption mass spectral analysis to determine the number of haptens.

EXAMPLE 7

Assays to Detect the Function Activity of CTB

To test the functional activity of CTB alone, two assays were developed. First, binding of CTB to cells was measured using flow cytometry. Cells were incubated with CTB, followed by a commercial anti-CTB goat antiserum and a fluorescein isothiocyanate (FITC)-labelled anti-goat secondary antibody (Figure 13). Native pentameric CTB bound to the cells, causing a dramatic shift in fluorescence intensity. Monomeric CTB was unable to bind to cells in this assay. Second, an ELISA was set up to measure the ability of the CTB to bind to ganglioside GM1. ELISA plates were coated with GM1-ganglioside and incubated with yarying concentrations of CTB. Binding was detected using an anti-CTB antibody (or saline as a control) followed by enzyme-labelled second antibody and development with substrate. This assay provided a quantitative and extremely sensitive measure of the ability of pentameric CTB to bind to GM1 gangliosides. These assays are used to monitor the functional activity of recombinant and haptenated CTB conjugates prior to experiments *in vivo*.

EXAMPLE 8

Co-Treatment with Other Drugs

With respect to treatment of heroin abuse, screening is done to determine whether pharmacotherapy with a second drug will enhance the activity of the therapeutic vaccine. Treatment with opiate antagonists, such as naloxone and naltrexone, and other antagonists, such as nalorphine, levallo[han, cyclazocine,

buprenorphine and pentazocine are expected to be compatible with treatment with a heroin conjugate. It is possible that one or more of the therapeutic agents could be immunosuppressive, thus inhibiting the induction of a high titer anti-heroin antibody response. To address this possibility, rats are immunized with the heroin-carrier conjugate in the presence or absence of the co-therapeutic drug and the antibody titer is measured at varying times. A co-therapeutic drug which is found to be significantly immunosuppressive will be eliminated as an incompatible therapy. This screening test is used for any drug for which co-treatment is considered.

If no immunosuppression is seen, further screening is carried out to determine if the two approaches synergize. Following training, immunization and testing, rats are further evaluated in the two models in the presence of the drugs. Rats will receive drugs before sessions with different doses of heroin. Initial experiments with control carrier-immunized rats are performed to choose a dose of drug that does not completely extinguish behavior in the self-administration or drug discrimination systems. Data is evaluated to determine whether the action of the therapeutic vaccine is additive with the co-therapeutic treatment.

EXAMPLE 9

Induction of Mucosal Response

The B subunit of cholera toxin (CTB) has been shown in many systems to retain the activity of intact cholera toxin, including the induction of a mucosal antibody response. Therefore, this carrier should induce a strong anti-heroin or anti-nicotine IgA antibody response. In addition, oral priming should induce a strong systemic IgG antibody response.

An effective way to prime an immune response in the respiratory tract is to deliver antigen directly to those sites. The antigen is administered in saline, with CTB acting as its own adjuvant. To confirm the ability of CTB to prime by administration at a mucosal IgA surface, initial experiments are conducted with

carrier alone. Mice are primed with 50 μg of the CTB or heroin-CTB or nicotine-CTB conjugate by three routes: orally, nasally or intratracheally. For oral administration of mice, 250 μg of either heroin-CTB or nicotine-CTB conjugate or CTB alone is applied intragastrically, or directly to the stomach, through the use of a blunt 23G needle. Fourteen days after priming, the mice are boosted using the same protocol. Nasal administration is a simple and common route of priming. Antigen is applied to each nostril of a lightly anesthetized mouse, for a total volume of 50 μ1 per mouse. Fourteen days after priming, the mice are boosted using the same protocol. Nasal administration is adaptable readily to human application as a nasal spray. Nasal vaccination has been used successfully with live influenza vaccines (Walker et al. (1994) *Vaccine* 12:387-399).

Intratracheal immunization directly applies the antigen to the lower respiratory tract, thereby enhancing immunity in the lungs. Mice are anesthetized with a cocktail of ketamine and xylazine. The animals are mounted on an apparatus that holds their mouth open and exposes the trachea; the trachea is visualized with a fiberoptic light probe. A blunt 23 gauge needle is used to deliver 50 μ1 of solution into the lungs. Fourteen days after priming, the mice are boosted using the same protocol.

Animals are sacrificed by CO_2 asphyxiation at varying time points after boost (14, 21, or 28 days) and nasal and bronchoalveolar lavage fluids are collected and assayed for IgA specific for the administered conjugate. Nasal wash fluid is obtained by washing the nasal cavity four times with a total of 1 ml PBS as described (Tamura et al. (1989) *Vaccine* 7:257-262). Bronchoalveolar lavage fluid is obtained by surgically exposing the trachea, injecting 0.5 ml PBS into the lungs, and rinsing three times as described (Nedrud et al. (1987) *J. Immunol.* 139:3484-3492). Following centrifugation to remove cells, samples are assayed for antigen-specific IgA by ELISA using an IgA-specific second antibody. Heroin-specific or nicotine-specific IgG is measured in the nasal and lung washes, as it has been reported that IgG is frequently both detectable and important in the lung (Cahill et al. (1993) *FEMS Microbiol. Lett.* 107:211-216).

The oral immunization route is evaluated for its ability to generate heroin-specific or nicotine-specific IgA in intestinal washes and is compared with other routes for its ability to generate serum Ig specific for heroin or nicotine. Oral administration is particularly preferred in humans due to ease of administration. The intranasal and intratracheal routes of administration are compared directly for their ability to induce an IgA response in both the lung or nasal lavage fluid. Whichever route is found to be most potent, it is preferred and used for the remaining experiments. If the two routes are of comparable efficacy, nasal immunization is preferred because of its simplicity.

For maximal protection against heroin or nicotine, systemic IgG and mucosal IgA responses may both be maximized. Therefore, both a systemic injection with the heroin-CTB or nicotine-CTB conjugate in alum (or some other adjuvant) and a mucosal challenge with the conjugate are preferred to effectively prime both compartments. Three groups are compared. First, mice are primed systemically, followed by a mucosal challenge after 14 days. Second, the mice are primed mucosally, followed by a systemic challenge after 14 days. Third, they are primed both systemically and mucosally at the same time, followed by an identical boost after 14 days. Control mice are primed only mucosally or only systemically. In each case, efficacy in challenge is determined by measurement of both IgG and IgA antibody titers.

As an initial measure of the *in vivo* efficacy of mucosal anti-heroin or anti-nicotine antibodies, the change in drug pharmacokinetics is measured for mucosally administered heroin or nicotine, respectively.

EXAMPLE 10

Passive Transfer of Immune Immunoglobulin in Mice

Mice are immunized with a heroin conjugate using optimal immunization regimens as described in the Examples. At varying times, mice are bled and the titers of anti-heroin antibody are assessed by ELISA. Animals with antibody titers

of about 54,000 or greater are sacrificed and bled by cardiac puncture. Control mice are immunized with the carrier protein alone. Sera from multiple mice (at least 20) are pooled and the IgG fraction is isolated by ammonium sulfate precipitation. Following dialysis to remove the ammonium sulfate, the level of cocaine-specific antibody in the pooled immunoglobulin fraction is quantified by ELISA. Varying amounts of immunoglobulin are administered i.p. or i.v. to naive mice. After 24 hours, the recipient mice are bled and the serum assayed to determine the level of cocaine-specific antibody. Using this method, the amount of antibody that must be transferred to achieve a given titer is determined. Groups of mice are given immune immunoglobulin and bled at varying periods of time to determine the clearance rate of the antigen-specific antibody. Other groups of mice are challenged with radiolabelled heroin, as described in the Examples, and heroin distribution to the brain are measured. Control mice received IgG from carrier-immunized mice. These experiments demonstrate the ability of passively transferred immune immunoglobulin to inhibit heroin entry into the brain.

EXAMPLE 11

Passive Transfer of Immune Immunoglobulin in Humans

A pool of human donors is immunized with a conjugate of the invention using optimal immunization regimens as described in the Examples. At various times, donors are bled by venipuncture and the titers of anti-haptent antibody are assayed by ELISA. Hyperimmune plasma from multiple donors is pooled and the IgG fraction is isolated by cold alcohol fractionation. The antibody preparation is buffered, stabilized, preserved and standardized as needed for hyperimmune antibody preparations for human use. The level of anti-hapten antibody is standardized by ELISA or other antibody-based assay.

An appropriate dose of purified antibody is administered to 20 patients intramuscularly or intravenously with or without the hapten-CTB vaccine, but not in the same anatomical site as the vaccine. The appropriate dose is determined by

assaying serum levels of recipients in a trial patient population by ELISA or other antibody-based assay at 24 hours or other appropriate time point after injection of the hyperimmune antibody preparation and/or assaying the effectiveness of different doses in inhibiting the effects of heroin or nicotine.

The passively transferred immune globulin inhibits the effects of heroin or nicotine in the patients. The use of human donors, polyclonal antibody, and the large number of donors in the donor pool limits the chance of immune response by the patients to the transferred antibody.

EXAMPLE 12

Preparative-Scale Purification of rCTB

rCTB from V. *cholerae* supplied from SBL Vaccin AB in 0.22 M phosphate pH 7.3, 0.9% NaCl buffer was diafiltered into 20 mM sodium phosphate, pH 6.5. A sample was then purified using cation exchange chromatography on Pharmacia SP Sepharose Fast Flow resin with Buffer A: 20 mM sodium phosphate pH 6.5 and Buffer B: 20 mM sodium phosphate pH 6.5, 1.0 M NaCl as the elution buffers. The purified fractions were analyzed by SDS-PAGE, staining with Daichi Silver Stain. The purified sample was filtered through a 0.22 micron filter and stored sterile at 4°C.

EXAMPLE 13

Method A (Analytical) Samples for analytical reverse phase HPLC (RP HPLC) were prepared by the following method: 100 μl of conjugate CTB-5.200 was precipitated by adding 1.0 ml of absolute ethanol and freezing at -80°C overnight. The conjugate was spun at 14000 rpm for 20 minutes at 4°C and then the ethanol was decanted off and the pellet air dried. The pellet was resuspended in 25 μl of 20% acetonitrile with 0.1% triflouroacetic acid (TFA) and protein concentration measured by the Pierce Micro BCA assay.

The conjugate was analyzed using a C18 reverse phase column (Vydac No. 218TP5215 narrow bore) 2.1 x 150mm; particle size: 5μ flow rate: 200 μl/min.; Buffer A: 100% water 0.1% TFA; Buffer B: 80% acetonitrile, 0.08% TFA. The gradient started at 16%, increased to 56% over a period of 50 minutes, increased to 80% at 60 minutes, and was held for 10 minutes.

Method B (Semi-Preparative) Samples for RP HPLC on the semi-preparative scale were prepared as follows: two vials of CTB-5.200 lyophile were resuspended in 20% acetonitrile 0.1% TFA, sterile filtered, and quantitated by the Pierce Micro BCA. Two injections of 1.24 mg each were made on a semi-preparative RP HPLC system using a C18 column (Vydac No. 218TP1520) 10 x 50 mm, particle size: 5 μl; flow rate: 1.8 ml/min; Buffer A: 0.1% TFA in water; Buffer B: 0.08% TFA in 80% acetonitrile. A stepwise gradient was used as follows: 20%B for 10 minutes, 35%B for 40 minutes, 55%B for 5 minutes, finishing with a 5 minute wash out at 100%B. Peaks were collected and immediately lyophilized.

EXAMPLE 14

Level of Haptenation vs. Immunogenicity

The ratio of drug hapten to carrier protein in the conjugate may alter the ability of the conjugates to stimulate production of hapten-specific antibody. The conjugation reaction is altered to produce heroin-CTB conjugates with several different levels of haptenation. Degree of haptenation is calculated by analysis of mass spectrometry of the conjugates. The conjugates were screened for biological activity in immunogenicity experiments and by mass spectrometry analysis for haptenation levels. Conjugates made by different methods using different ratios of haptenation reagents compared to carrier protein. A comparison of level of haptenation and immunogenicity is made.

Equivalents

Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, numerous equivalents to the specific substances and procedures described herein. Such equivalents are considered to be within the scope of this invention, and are covered by the following claims.

What is claimed is:

1. A hapten-carrier conjugate comprising the structure shown in Figure 6b, wherein A, B, C, D, E and F are branches off a nicotine molecule, and are each selected from the group of chemical moieties identified by CJ reference number, consisting of:

CJ 0	Q
CJ 1	$(CH_2)_nQ$
CJ 1.1	CO_2Q
CJ 1.2	COQ
CJ 1.3	OCH_3
CJ 2	$OCO(CH_2)_nQ$
CJ 2.1	OCOCH=Q
CJ 2.2	$OCOCH(O)CH_2$
CJ 2.3	$OCO(CH_2)_nCH(O)CH_2$
CJ 3	$CO(CH_2)_nCOQ$
CJ 3.1	$CO(CH_2)_nCNQ$
CJ 4	$OCO(CH_2)_nCOQ$
CJ 4.1	$OCO(CH_2)_nCNQ$
CJ 5	$CH_2OCO(CH_2)_nCOQ$
CJ 5.1	$CH_2OCO(CH_2)_nCNQ$
CJ 6	$CONH(CH_2)_nQ$
CJ 7	$Y(CH_2)_nQ$
CJ 7.1	$CH_2Y(CH_2)_nQ$
CJ 8	$OCOCH(OH)CH_2Q$
CJ 8.1	$OCO(CH_2)_nCH(OH)CH_2Q$
CJ 9	$OCOC_6H_5$
CJ 10	See Figure 1b
CJ 11	$YCO(CH_2)_nCOQ$

wherein Y is sulfur (S), oxygen (O), or an amine (NH), wherein n is an integer from 2 to 20, and wherein Q is selected from the group consisting of: H, OH, OCH3, CH2, CH3, COOH, halogens, activated esters, mixed anhydrides, acyl halides, acyl azides, alkyl halides, N-maleimides, imino esters, isocyanate, isothiocyanate, and a T cell epitope-containing carriers; with the proviso that Q in at least one of A, B, C, D, E, or F comprises at least one T cell epitope containing carrier, and the conjugate, with the exception of the T cell epitope-containing carrier, is referred to herein as the hapten.

2. The hapten-carrier conjugate of claim 1 wherein at least one hapten is coupled to the carrier.

3. The hapten-carrier conjugate of claim 2 wherein at least two haptens are coupled to the carrier, and the haptens are the same.

4. The hapten-carrier conjugate of claim 2 wherein the carrier is multivalent.

5. The conjugate of claim 1 wherein the T cell epitope-containing carrier is selected from the group consisting of a T cell epitope-containing protein, a modified T cell epitope-containing protein, a T cell epitope-containing peptide, a modified T cell epitope-containing peptide, a T cell epitope-containing peptidomimetic, T cell epitope-containing multiantigenic peptides (MAP), and at least one of the chemical moieties being identified by the CJ reference number and comprising at least one T cell epitope.

6. The conjugate of claim 5 wherein the T cell epitope-containing carrier is selected from the group consisting of cholera toxin B (CTB), diphtheria toxin, tetanus toxoid, pertussis toxin, pertussis filamentous hemagglutinin, shiga toxin, ricin B subunit, abrin, sweet pea lectin, retrovirus nucleoprotein (retro NP), rabies ribonucleoprotein (rabies RNP), Tobacco Mosaic Virus (TMV), cow pea mosaic virus, cauliflower mosaic virus, vesicular stomatitis virus-nucleocapsid protein (VSV-N), recombinant pox virus subunits and

vectors, Semliki forest virus vectors, Pseudomonas endotoxin, multiantigenic peptides (MAP), yeast virus-like particles (VPLs), malarial protein antigen, and microspheres.

7. The conjugate of claim 6 wherein the T cell epitope-containing carrier comprises cholera toxin B (CTB).

8. The conjugate of claim 6 wherein said T cell epitope containing carrier is selected from the group consisting of cholera toxin B (CTB), retrovirus nucleoprotein (retro NP), rabies ribonucleoprotein (rabies RNP) vesicular stomatitis virus-nucleocapsid protein (VSV-N), recombinant small pox virus subunit and vectors, and multiantigenic peptides (MAP).

9. The conjugate of claim 8 wherein MAP comprises a defined T cell epitope-containing peptide which is further conjugated to a multi-haptenated lysine branch structure at the amino terminus end of the peptide.

10. A hapten-carrier conjugate selected from the group consisting of PS-51, PS-52, PS-53, PS-54, PS-55, PS-56, PS-57, PS-58, PS-59 and PS-60 as depicted in Figure 7.

11. The hapten-carrier conjugate of claim 10 wherein at least one hapten is coupled to the carrier.

12. The hapten-carrier conjugate of claim 10 wherein at least two haptens are coupled to the carrier, and the haptens are the same.

13. The hapten-carrier conjugate of claim 11 wherein the carrier is multivalent.

14. A therapeutic composition comprising at least one conjugate of claim 1 and a pharmacologically acceptable excipient.

15. A therapeutic composition comprising at least one conjugate of claim 6 and a pharmaceutically acceptable carrier.

16. The therapeutic composition of claim 15 further comprising an adjuvant.

17. A therapeutic composition comprising at least one conjugate of claim 10 and a pharmaceutically acceptable carrier.

18. The therapeutic composition of claim 17 further comprising an adjuvant.

19. The therapeutic composition of claim 16 wherein the adjuvant is alum, MF59 or RIBI adjuvant.

20. The therapeutic composition of claim 15 wherein the composition is soluble in an aqueous solution at a physiological acceptable pH.

21. A method of treating drug addiction to nicotine in mammals comprising administering a therapeutically effective amount of the therapeutic composition of claim 15 to a mammal in need of treatment for drug addiction.

22. A method of treating drug addiction to nicotine in mammals comprising administering a therapeutically effective amount of a composition of claim 16 to a mammal in need of treatment for drug addiction.

23. A method of treating drug addiction to nicotine in mammals comprising administering a therapeutically effective amount of the therapeutic composition of claim 17 to a mammal in need of treatment for drug addiction.

24. A method of treating drug addiction to nicotine in mammals comprising administering a therapeutically effective amount of a composition of claim 18 to a mammal in need of treatment for drug addiction.

25. A method for treating drug addiction in a mammal comprising administering to an affected mammal an antibody specific for the hapten-carrier conjugate of claim 1.

26. The method of claim 25 wherein said antibody is selected from the group consisting of a polyclonal antibody and a monoclonal antibody.

27. The method of claim 25 wherein the antibody is specific for the hapten portion of the conjugate of claim 1.

28. A method for treating drug addiction in a mammal comprising administering to an affected mammal an antibody specific for the hapten component of the hapten-carrier conjugate of claim 10.

29. An antibody produced in response to the conjugate of claim 1.

30. The antibody of claim 29 selected from the group consisting of a polyclonal antibody and a monoclonal antibody.

31. The antibody of claim 29 which is specific for the hapten portion of the conjugate of claim 1.

32. The therapeutic composition of claim 15 wherein the composition is in suspension in an aqueous solution at a physiologically acceptable pH.

33. A method of preventing addiction to a drug in a mammal, said method comprising:

(a) administering an effective amount of the conjugate of claim 7 to said mammal;

(b) monitoring the mammal for a desired preventative effect, wherein production of anti-drug antibodies is an indication of the desired preventative effect.

34. A method of preventing addiction to a drug in a mammal, said method comprising:

(a) administering an effective amount of the therapeutic composition of claim 15 to said mammal;

(b) monitoring the mammal for a desired preventative effect, wherein production of anti-drug antibodies is an indication of the desired preventative effect.

35. The method according to claim 19 wherein the administration is enteral or parenteral.

36. The method according to claim 35 wherein the administration is oral or intramuscular

37. A hapten-carrier conjugate selected from the group consisting of PS-61, PS-62, PS-63, and PS-64

38. The hapten-carrier conjugate of claim 37 wherein at least one hapten is coupled to the carrier.

39. The hapten-carrier conjugate of claim 38 wherein at least two haptens are coupled to the carrier, and the haptens are the same.

40. The hapten-carrier conjugate of claim 38 wherein the carrier is multivalent.

41. The conjugate of claim 37 wherein the T cell epitope-containing carrier is selected from the group consisting of a T cell epitope-containing protein, a modified T cell epitope-containing protein, a T cell epitope-containing peptide, a modified T cell epitope-containing peptide, a T cell epitope-containing peptidomimetic, T cell epitope-containing multiantigenic peptides (MAP), and at least one of the chemical moieties being identified by the CJ reference number and comprising at least one T cell epitope.

42. The conjugate of claim 41 wherein the T cell epitope-containing carrier is selected from the group consisting of cholera toxin B (CTB), diphtheria toxin, tetanus toxoid, pertussis toxin, pertussis filamentous hemagglutinin, shiga toxin, ricin B subunit, abrin, sweet pea lectin, retrovirus nucleoprotein (retro NP), rabies ribonucleoprotein (rabies RNP), Tobacco Mosaic Virus (TMV), cow pea mosaic virus, cauliflower mosaic virus, vesicular stomatitis

virus-nucleocapsid protein (VSV-N), recombinant pox virus subunits and vectors, Semliki forest virus vectors, Pseudomonas endotoxin, multiantigenic peptides (MAP), yeast virus-like particles (VPLs), malarial protein antigen, and microspheres.

43. The conjugate of claim 42 wherein the T cell epitope-containing carrier comprises cholera toxin B (CTB).

44. The conjugate of claim 42 wherein said T cell epitope containing carrier is selected from the group consisting of cholera toxin B (CTB), retrovirus nucleoprotein (retro NP), rabies ribonucleoprotein (rabies RNP) vesicular stomatitis virus-nucleocapsid protein (VSV-N), recombinant small pox virus subunit and vectors, and multiantigenic peptides (MAP).

45. The conjugate of claim 44 wherein MAP comprises a defined T cell epitope-containing peptide which is further conjugated to a multi-haptenated lysine branch structure at the amino terminus end of the peptide.

46. A therapeutic composition comprising at least one conjugate of claim 37 and a pharmacologically acceptable excipient.

47. The therapeutic composition of claim 46 further comprising an adjuvant.

48. The therapeutic composition of claim 47 wherein the adjuvant is alum, MF59 or RIBI adjuvant.

49. A method of treating drug addiction to heroin in mammals comprising administering a therapeutically effective amount of the therapeutic composition of claim 46 to a mammal in need of treatment for drug addiction.

50. The method according to claim 49 wherein the adminstration is enteral or parenteral.

51. An antibody produced in response to the conjugate of claim 37.

52. The antibody of claim 51 selected from the group consisting of a polyclonal antibody and a monoclonal antibody.

53. A method for treating drug addiction to heroin in a mammal comprising administering to an affected mammal an antibody specific for the hapten portion of the conjugate of claim 37.

54. The method of claim 53 wherein said antibody is selected from the group consisting of a polyclonal antibody and a monoclonal antibody.

FIG. 1a

CJ #	Branch	Variables
CJ 0	Q	Q = H, OH, CH_2, halogen, COOH, carrier protein, modified carrier protein
CJ 1	$(CH_2)_nQ$	Q = H, COOH, halogen, 2-nitro-4-sulfophenyl ester, N-oxysuccinimidyl ester, carrier protein, modified carrier protein, CJ 1.2
CJ 1.1	CO_2Q	Q = H, CH_3
CJ 1.2	COQ	Q = H, halogen, 1-oxy-2-nitro-4-sulfophenyl, N-oxysuccinimidyl, N-maleimidyl, carrier protein, CJ 10
CJ 2	$OCO(CH_2)_nQ$	Q = COOH; halogen, 2-nitro-4-sulfophenyl ester, N-oxysuccinimidyl ester, carrier protein, modified carrier protein
CJ 2.1	OCOCH=Q	Q = H
CJ 2.2	$OCOCH(O)CH_2$	
CJ 2.3	$OCO(CH_2)_nCH(O)CH_2$	
CJ 3	$CO(CH_2)_nCOQ$	Q = H, OH, halogen, 1-oxy-2-nitro-4-sulfophenyl, N-oxysuccinimidyl, N-maleimidyl, carrier protein, CJ 10
CJ 3.1	$CO(CH_2)_nCNQ$	Q = OCH_3 or carrier protein
CJ 4	$OCO(CH_2)_nCOQ$	Q = H, OH, halogen, 1-oxy-2-nitro-4-sulfophenyl, N-oxysuccinimidyl, N-maleimidyl, carrier protein, CJ 10
CJ 4.1	$CO(CH_2)_nCNQ$	Q = OCH_3 or carrier protein
CJ 5	$CH_2OCO(CH_2)_nCOQ$	Q = H, OH, halogen, 1-oxy-2-nitro-4-sulfophenyl, N-oxysuccinimidyl, N-maleimidyl, carrier protein, CJ 10
CJ 5.1	$CO(CH_2)_nCNQ$	Q = OCH_3 or carrier protein
CJ 6	$CONH(CH_2)_nQ$	Q = H, COOH, halogen, 2-nitro-4-sulfophenyl ester, N-oxysuccinimidyl ester, carrier protein, modified carrier protein
CJ 7	$Y(CH_2)_nQ$	Y = S, O, NH; Q = halogen, COOH, carrier protein, modified carrier protein
CJ 7.1	$CH_2Y(CH_2)_nQ$	Y = S, O, NH; Q = halogen, COOH, carrier protein, modified carrier protein
CJ 8	$OCOCH(OH)CH_2Q$	Q = carrier protein, modified carrier protein
CJ 8.1	$OCO(CH_2)_nCH(OH)CH_2Q$	Q = carrier protein, modified carrier protein
CJ 9	$OCOC_6H_5$	
CJ 11	$YCO(CH_2)_nCOQ$	Y = S, O, NH; Q = OH, carrier protein, modified carrier protein or halogen

ALTERNATIVE REPRESENTATION FOR SELECTED BRANCHES

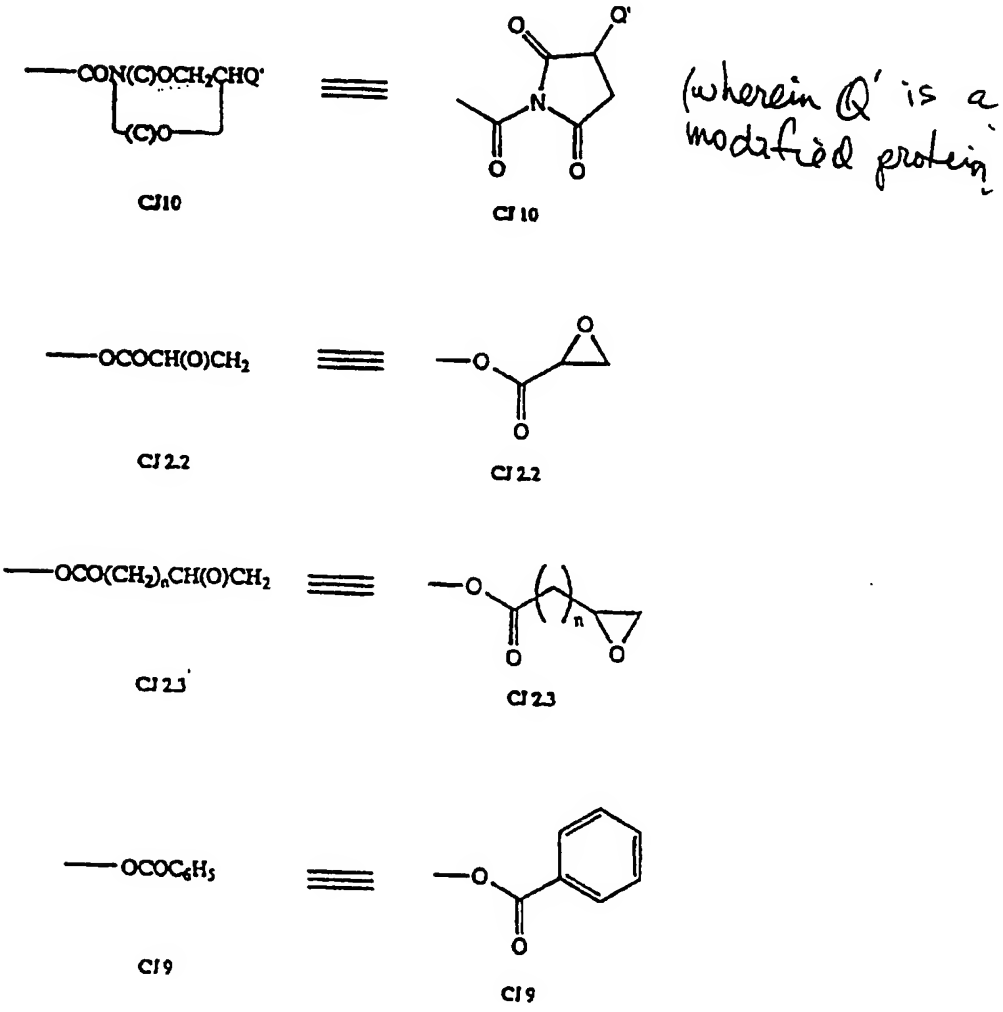
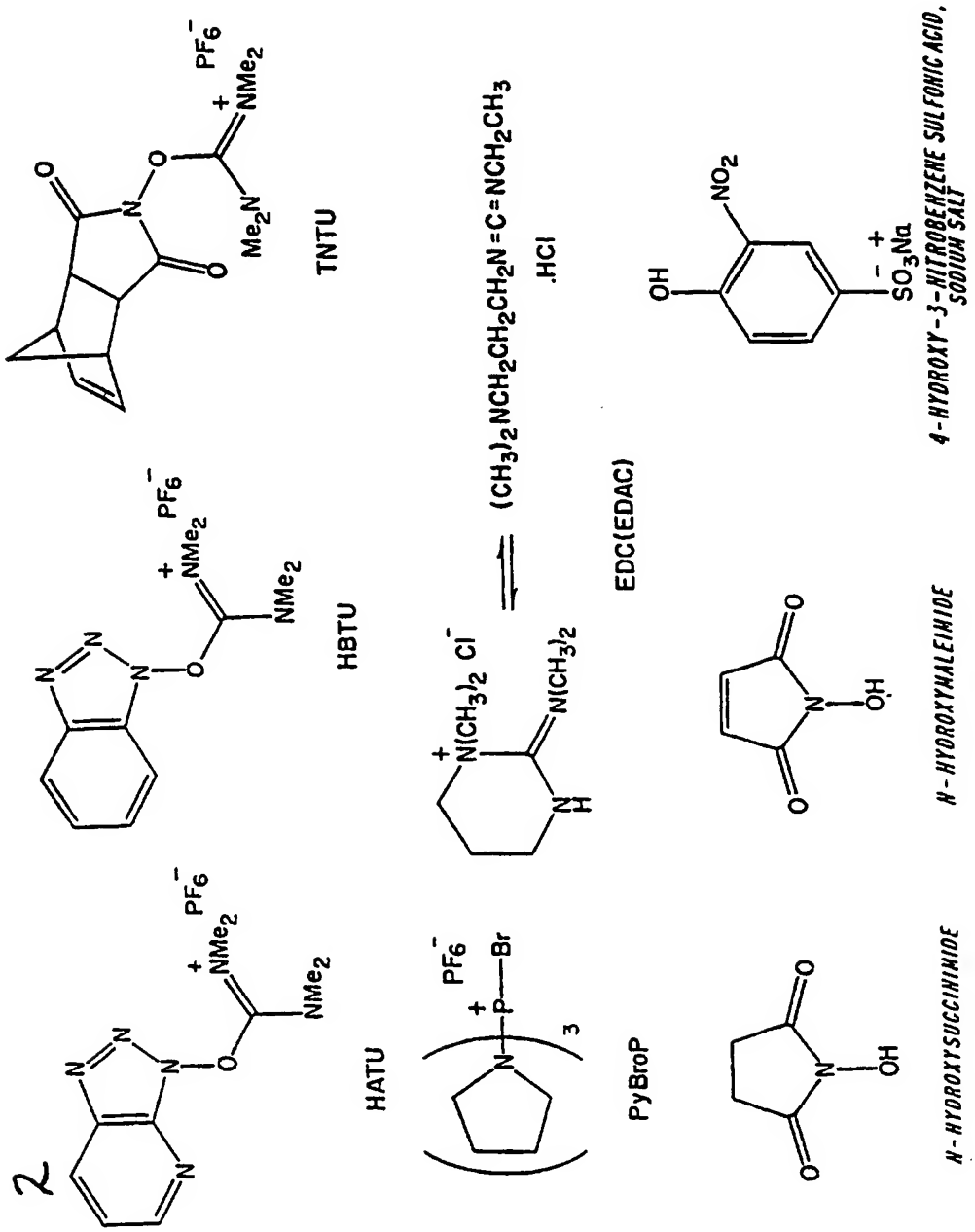
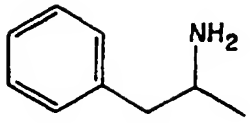


FIG. 1b

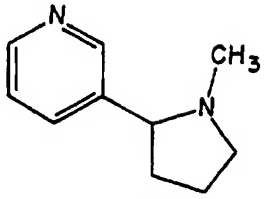
FIG. 2


TNTU
HBTU
HATU
PyBroP
EDC(EDAC)
.HCl
4-HYDROXY-3-NITROBENZENE SULFONIC ACID, SODIUM SALT
N-HYDROXYMALEIMIDE
N-HYDROXYSUCCINIMIDE

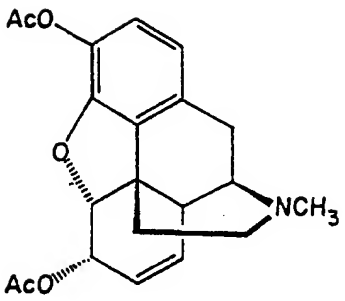
OTHER COMMONLY ABUSED DRUGS



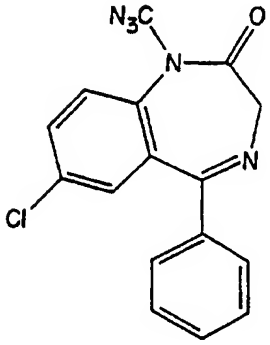
AMPHETAMINE



NICOTINE



HEROIN



DIAZEPAM

FIG. 3

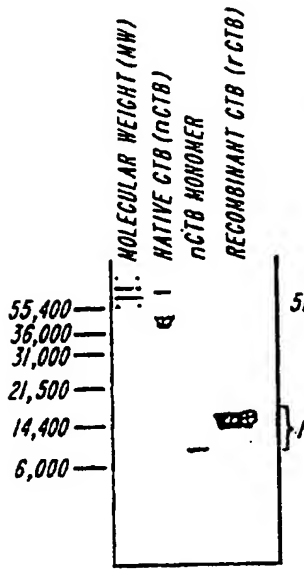


FIG. 4 A

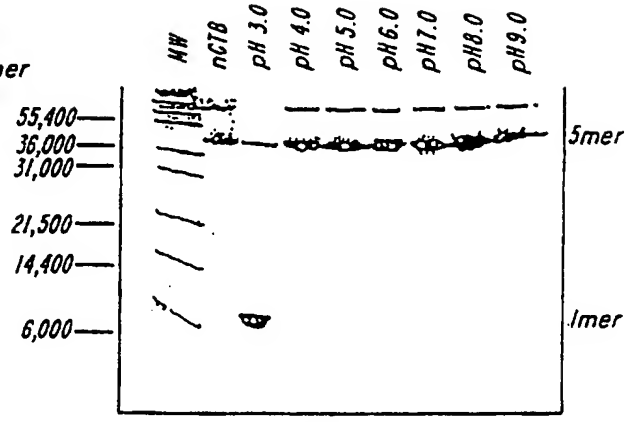


FIG. 4 B

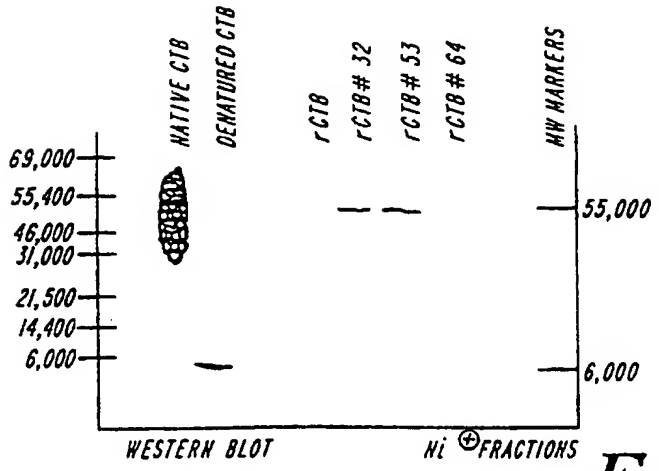


FIG. 4 C

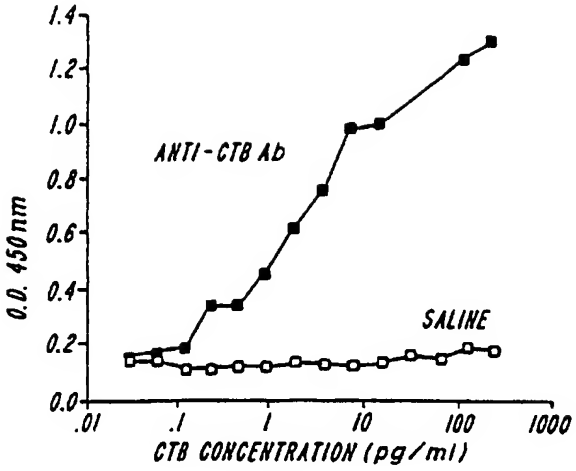

1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
O.D. 450nm
ANTI-CTB Ab
SALINE
.01
.1
1
10
100
1000
CTB CONCENTRATION (pg/ml)

FIG. 5A

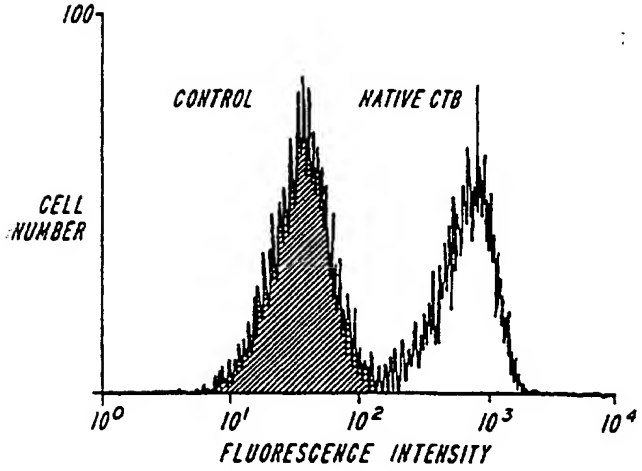

100
CONTROL
NATIVE CTB
CELL NUMBER
10^0
10^1
10^2
10^3
10^4
FLUORESCENCE INTENSITY

FIG. 5B

Figure 6a

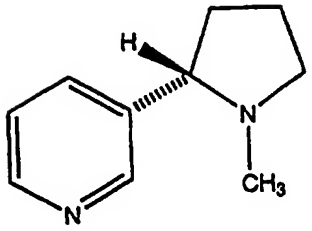
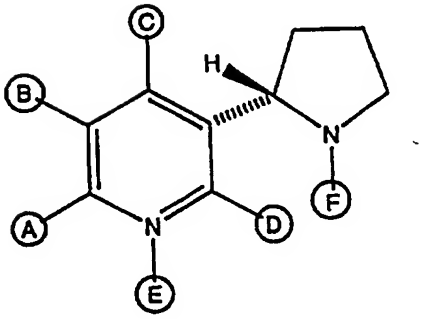


Figure 6b



	A	B	C	D	E	F
PS-51	CJ1 where Q=COOH and n=2-8, preferably n=4 or where Q=CJ1.2, wherein Q=T-cell epitope containing carrier.	CJO where Q=H	CJO where Q=H	CJO where Q=H	—	CJO where Q=CH_3
PS-52	CJO where Q=H	CJ1 where Q=COOH and n=2-8, preferably n=4 or where Q=CJ1.2, wherein Q=T-cell epitope containing carrier.	CJO where Q=H	CJO where Q=H	—	CJO where Q=CH_3
PS-53	CJO where Q=H	CJO where Q=H	CJ1 where Q=COOH and n=2-8, preferably n=4 or where Q=CJ1.2, wherein Q=T cell epitope containing carrier.	CJO where Q=H	—	CJO where Q=CH_3
PS-54	CJO where Q=H	CJO where Q=H	CJO where Q=H	CJO where Q=H	—	CJ3 where Q=OH or T cell epitope containing carrier, n=2
PS-55	CJO where Q=H	CJO where Q=H	CJO where Q=H	CJO where Q=H	CJ1 where Q=H or a Tcell epitope containing carrier n=3	CJO where Q=CH_3
PS-56	CJO where Q=H	CJO where Q=H	CJO where Q=H	CJO where Q=H	CJ1 where Q=H or a Tcell epitope containing carrier n=4	CJO where Q=CH_3

Figure 7a

	A	B	C	D	E	F
PS-57	CJO where Q=H	CJO where Q=H	CJO where Q=H	CJO where Q=H	CJ1 where Q=H or a Tcell epitope containing carrier n=5	CJO where Q=CH_3
PS-58	CJO where Q=H	CJO where Q=H	CJO where Q=H	CJO where Q=H	CJ1 where Q=H or a Tcell epitope containing carrier n=7	CJO where Q=CH_3
PS-59	CJ11 where Y=NH Q=OH or T-cell epitope containing carrier where n=2-8, preferably n=3	CJO where Q=H	CJO where Q=H	CJO where Q=H	—	CJO where Q=CH_3
PS-60	CJO where Q=H	CJO where Q=H	CJO where Q=H	CJO where Q=H	—	CJ1 where Q=COOH or where Q=CJ1.2, wherein Q=T-cell epitope containing carrier, where n=2-8, preferably n=4

Figure 7b

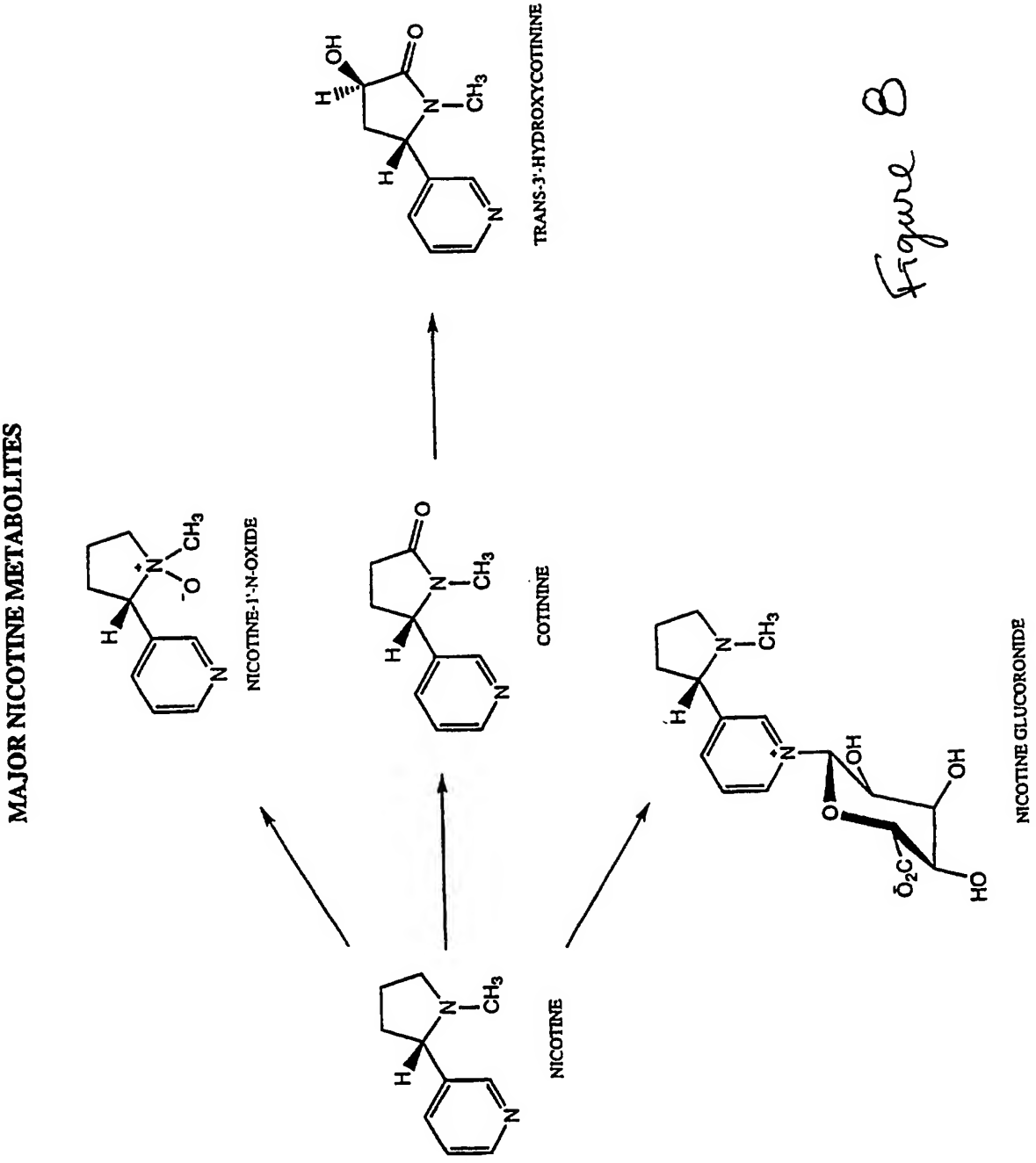

MAJOR NICOTINE METABOLITES
NICOTINE-1'-N-OXIDE
COTININE
TRANS-3'-HYDROXYCOTININE
NICOTINE
NICOTINE GLUCORONIDE

Figure 8

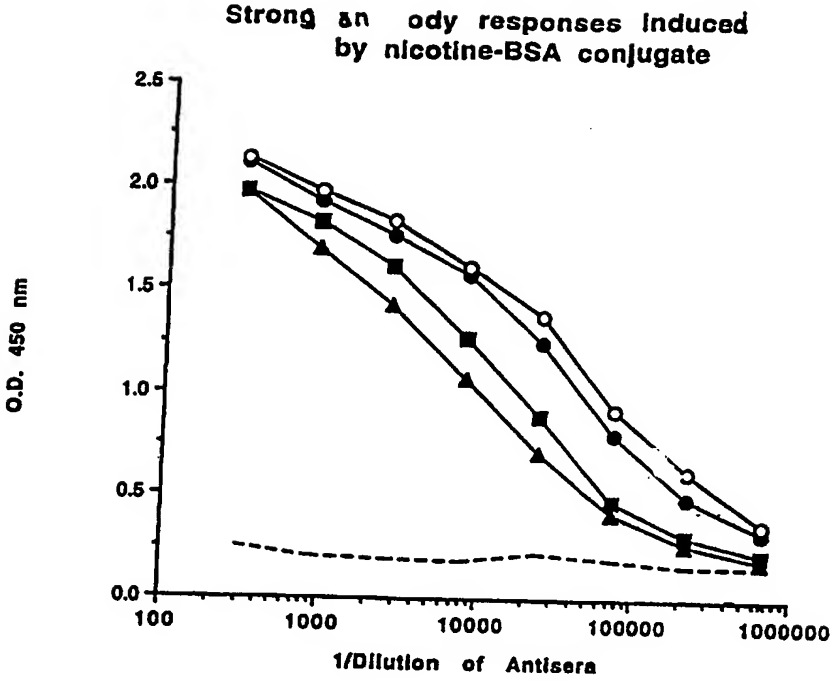

Strong an ody responses induced
by nicotine-BSA conjugate
O.D. 450 nm
2.5
2.0
1.5
1.0
0.5
0.0
100
1000
10000
100000
1000000
1/Dilution of Antisera

Fig. 9a

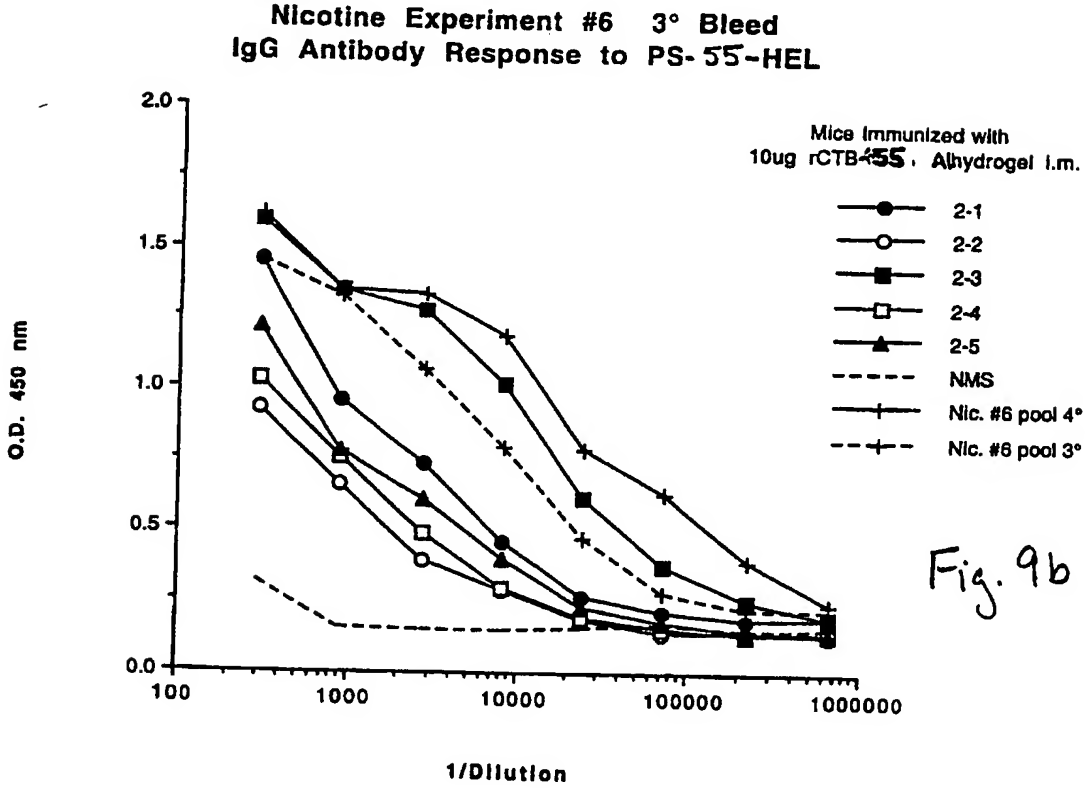

Nicotine Experiment #6 3° Bleed
IgG Antibody Response to PS-55-HEL
O.D. 450 nm
2.0
1.5
1.0
0.5
0.0
100
1000
10000
100000
1000000
1/Dilution
Mice immunized with
10ug rCTB-55, Alhydrogel i.m.
2-1
2-2
2-3
2-4
2-5
NMS
Nic. #6 pool 4°
Nic. #6 pool 3°

Fig. 9b

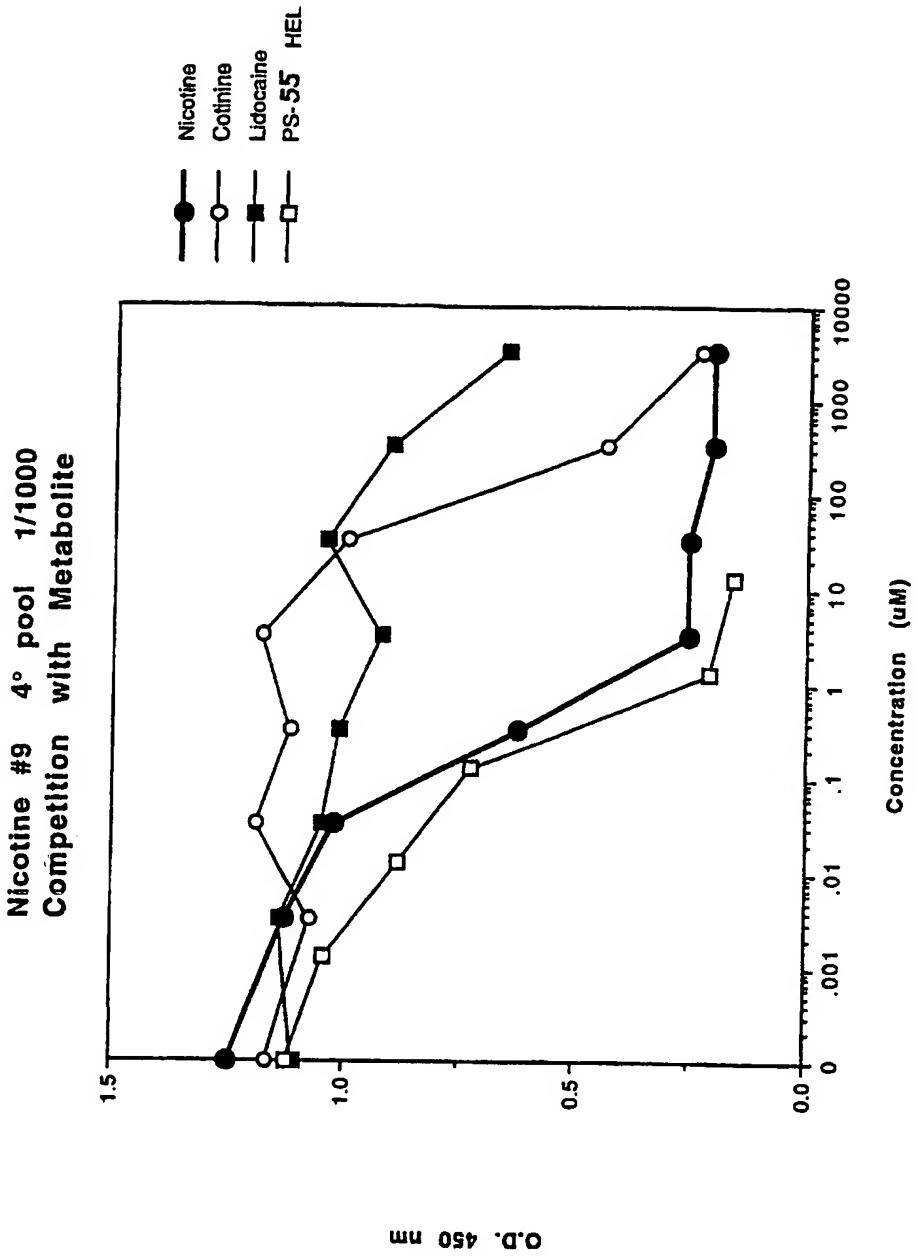
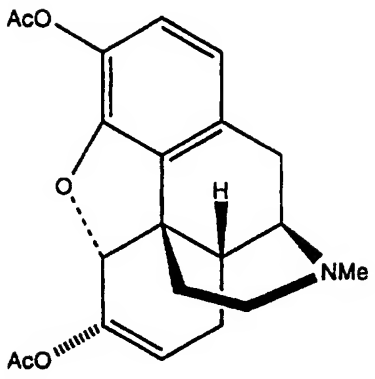

Nicotine #9 4° pool 1/1000
Competition with Metabolite
Nicotine
Cotinine
Lidocaine
PS-55 HEL
O.D. 450 nm
1.5
1.0
0.5
0.0
0
.001
.01
.1
1
10
100
1000
10000
Concentration (uM)

Figure 10

Figure 11a



HEROIN

Figure 11b

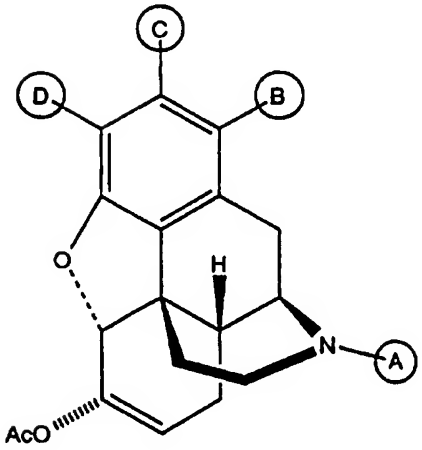


Figure 12

	A	B	C	D
PS-61	CJ1 where Q=COOH or where Q=CJ1.2 and n=2-8, preferably n=4 wherein Q=T-cell epitope containing carrier	CJ0 where Q=H	CJ0 where Q=H	CJ2 where Q=H and n=1
PS-62	CJ0 where Q=CH_3	CJ0 where Q=H	CJ1 where Q=COOH or where Q=CJ1.2 and n=2-8, preferably n=4 wherein Q=T-cell epitope containing carrier	CJ2 where Q=H and n=1
PS-63	CJ0 where Q= CH_3	CJ1 where Q=COOH or where Q=CJ1.2 and n=2-8, preferably n=4 wherein Q=T-cell epitope containing carrier	CJ0 where Q=H	CJ2 where Q=H and n=1
PS-64	CJ0 where Q= CH_3	CJ0 where Q=H	CJ0 where Q=H	CJ4 where n=2 and Q=OH or Q=T-cell epitope containing carrier